



GNE.3230R1C112

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Goddard, et al. (as amended herein)
Appl. No.	:	10/063,670
Filed	:	May 7, 2002
For	:	SECRETED AND TRANSMEMBRANE POLYPEPTIDES AND NUCLEIC ACIDS ENCODING THE SAME
Examiner	:	Gary B. Nickol
Group Art Unit	:	1642

DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. This Declaration is being submitted to demonstrate that nucleic acids which encode polypeptides which enhance TNF- α levels are therapeutically useful.
2. I am an inventor on the above-identified patent application and am familiar with the specification and prosecution history.
3. I have extensive experience in the field of the claimed invention as indicated in the attached Curriculum Vitae provided herewith as Exhibit A. ✓
4. The claims in the above-identified application relate to polynucleotides encoding polypeptides which stimulate TNF- α release from human blood.
5. Polynucleotides which encode polypeptides which can be used to enhance TNF- α levels are therapeutically beneficial. As discussed in the following paragraph, as of October 29, 1997, the filing date of the earliest application to which the present application claims priority, it was known that increasing TNF- α levels by direct administration of TNF- α ameliorates several medical conditions. The same therapeutic benefits which are achieved through direct administration of TNF- α can be achieved by indirectly increasing TNF- α levels using the polypeptides encoded by the claimed polynucleotides, which induce the release of TNF- α .

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TNF- α was originally identified as a polypeptide which had antitumor properties. (See Carswell, E.A. et al. Proc Natl. Acad. Sci. 25: 3666-3670 (1975), attached hereto as Exhibit B). The antitumor effects of TNF- α were subsequently verified and exploited in several contexts. For example, Hallahan et al. demonstrated that adenoviral vectors comprising the TNF- α gene were successful in treating tumors in animals. (See Hallahan et al., Nat. Med. 1: 786-791 (1995), attached hereto as Exhibit C). TNF- α was also shown to induce necrosis of transplanted tumors, to have cytotoxic properties, and to have anti-viral properties (See Goeddel, D.V. et al. Cold Spring Harbor Symposia on Quantitative Biology 51:597-609 (1986), attached hereto as Exhibit D). In addition, TNF- α and other cytokines were known to protect against ionizing radiation in the context of radiotherapy. (See Neta et al., J. Immunol 136(7) 2483 (1987), attached hereto as Exhibit E).

In addition to the foregoing scientific literature relating to the therapeutic benefits associated with administering TNF- α , numerous patents which relate to the use of TNF- α as a therapeutic agent alone or in conjunction with other therapeutically active agents had issued prior to October 29, 1997, including U.S. Patent Nos.: 5,215,743 (stabilized TNF compositions); 5,059,530 (expression vectors encoding TNF); 4,894,225 (therapeutic use of TNF in conjunction with immunotoxin); 4,980,160 (therapeutic use of TNF in conjunction with non-steroidal anti-inflammatory agents); and 4,963,354 (use of TNF as an adjuvant in combination with a substance against which it is desired to raise an immune response). Each of these patents is attached hereto as Exhibits F-J.

The claimed polynucleotides can be used to produce their encoded polypeptides. Since these polypeptides stimulate release of TNF- α , thereby increasing TNF- α levels, they can achieve the same therapeutic benefits which result from direct administration of TNF- α and which are described in the foregoing references. Accordingly, polynucleotides encoding polypeptides which can be used to enhance which induce the release of TNF- α levels are therapeutically useful.

In addition to the therapeutic benefits resulting from using the polypeptides encoded by the claimed polynucleotides to enhance TNF- α levels discussed above, there are other therapeutic contexts in which the encoded polypeptides can be used to achieve therapeutic benefits by decreasing TNF- α levels. As of October 29, 1997, it was known that there were

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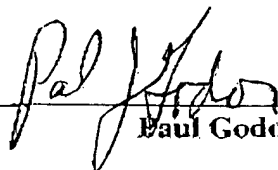
several medical conditions which can be ameliorated by reducing TNF- α levels. In particular, reducing TNF- α levels has been shown to be beneficial in treating conditions such as rheumatoid arthritis and Crohn's disease. (See Paleolog, E. Mol. Pathol. 1997, 50: 225-233 (1995) and Eigler, A. et al., Immunol. Today 18:487-492(1997), attached hereto as Exhibits K and L.

In addition, prior to October 29, 1997 numerous patents which relate to the therapeutic benefits of blocking the activity of TNF- α had issued, including U.S. Patent Nos.: 5,436,154 (antibodies which bind to and neutralize TNF- α) and 5,656,272 (methods of treating Crohn's disease using chimeric antibodies against TNF- α), attached hereto as Exhibits M and N.

The claimed polynucleotides can be used to produce their encoded polypeptides. In turn, the polypeptides can be used to generate antibodies which neutralize the activity of the polypeptides. Such antibodies can be used to achieve the therapeutic benefits resulting from reducing TNF- α levels which are described in the foregoing references. Accordingly, polynucleotides which encode polypeptides which stimulate the release of TNF- α are therapeutically useful.

7. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated: 11/24/01

By: 
Paul Godowski

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PROFESSIONAL HISTORY

August, 2003- Present	Staff Scientist, Department of Immunology (Joint Appointment with Department of Molecular Biology) Genentech, Inc South San Francisco, CA 94080
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EDUCATION

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JOB RESPONSIBILITIES

As a Director and Senior Director of Research, I supervised the activities of up to 160 employees in the Departments of Molecular Biology, Cell Biology, Protein Chemistry, Bioinformatics and Assay & Automation Technology within the Technology Branch of Genentech Research. I reported directly to the Senior Vice President of Research. The responsibilities of these Departments were broad, extending from early stage Research through support of Development projects. The major effort in the from 1996-2001 focused on Genomics, in particular the identification, cloning, sequencing, expression and functional analysis of several thousand human genes encoding secreted proteins and transmembrane receptors. The functional areas covered within these Departments included development of programs to search EST and Genomic Databases for genes based on homology, structure or other features of interest, database design for all of Research, the DNA microarray facility (in house microarray efforts and

commercial arrays (Affymetrix, Agilent), all aspects of microarray data analysis and mining, DNA sequencing, protein expression (mammalian, baculovirus, and bacterial), protein purification, functional analysis of novel cytokines and receptors, high throughput cell-based assays, development of antibody-based assays, evaluation and support of robotic equipment for Research and Development, and the Research FACS and Confocal Microscopy facilities. We worked closely to coordinate our efforts with Research Discovery Departments. As a member of the Research Review Committee (composed of 6 representatives from Research) I set strategic and tactical direction for all projects in Research.

The goal of our genomic program at Genentech was two-fold. The first goal was to identify and patent a large number of novel secreted proteins. The second goal was to develop a technological infrastructure within Research that would facilitate our capacity to turn those gene discoveries into drugs. Following the successful completion of these goals, I was promoted to Staff Scientist and established a Research Program to develop drugs for diseases with an Immunological basis as an out growth of my interest in the TLR receptor family. I currently head an effort to target the innate immune system, and in particular, myeloid cells, in autoimmune diseases.

In addition to my responsibilities within Research I also work closely with our Legal Department to establish our strategy for filing patents, with our Business Development Group to review in-licensing opportunities and structured our external Research contracts (for example, I was the lead person on our multimillion dollar contract with Gene Logic and Celera. I meet regularly with our Clinical Group to evaluate our Research directions. I often presented our programs to the Genentech Executive Committee, the Board of Directors, the Scientific Resource Board, and to many other functional areas within Genentech. I also represented Genentech at external Scientific and Financial forums.

Curriculum Vitae
Paul J. Godowski

PUBLICATIONS

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2. Godowski, P.J. and Knipe, D.M. (1983) Mutations in the major DNA-binding protein of herpes simplex virus type 1 result in increased levels of viral gene expression. J. of Virol. 47:478-486.
3. Godowski, P.J. and Knipe, D.M. (1985) Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. J. of Virol. 55:357-365.
4. Godowski, P.J. (1985) Regulation of herpes simplex virus type 1 gene expression. Ph.D. Thesis, Harvard University, Cambridge, Massachusetts.
5. Godowski, P.J. and Knipe, D.M. (1986) Transcriptional regulation of herpes simplex virus type 1 gene expression: Gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. U.S.A. 83:256-260.
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Patent Applications

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transmembrane proteins

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An endotoxin-induced serum factor that causes necrosis of tumors

(activated macrophage)

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ABSTRACT In studying "hemorrhagic necrosis" of tumors produced by endotoxin, it was found that the serum of bacillus Calmette-Guérin (BCG)-infected mice treated with endotoxin contains a substance (tumor necrosis factor; TNF) which mimics the tumor necrotic action of endotoxin itself. TNF-positive serum is as effective as endotoxin itself in causing necrosis of the sarcoma Meth A and other transplanted tumors. A variety of tests indicate that TNF is not residual endotoxin, but a factor released from host cells, probably macrophages, by endotoxin. Corynebacteria and Zymosan, which like BCG induce hyperplasia of the reticulo-endothelial system, can substitute for BCG in priming mice for release of TNF by endotoxin. TNF is toxic *in vitro* for two neoplastic cell lines; it is not toxic for mouse embryo cultures. We propose that TNF mediates endotoxin-induced tumor necrosis, and that it may be responsible for the suppression of transformed cells by activated macrophages.

One of the best-known enigmas of cancer biology is the "hemorrhagic necrosis" of tumors induced by endotoxin (1-5). We report here that endotoxin acts indirectly by causing the host to release a substance, which we name tumor necrosis factor (TNF), that is selectively toxic for malignant cells.

MATERIALS

BCG (bacillus Calmette-Guérin, Tice strain) was obtained from the Institute for Tuberculosis Research (University of Illinois Medical Center, Chicago, Ill.); *Corynebacterium granulosum* (Réticulostimuline, no. 90808) from the Pasteur Institute (Paris, France); *Corynebacterium parvum* from the Burroughs Wellcome Laboratories (Triangle Park, N.C.); Zymosan (from *Saccharomyces cerevisiae*) from Nutritional Biochemical Corporation; endotoxin (lipopolysaccharide W from *Escherichia coli*) from Difco (Detroit, Mich.); BCG old tuberculin (O.T.) from Jensen-Salisbury Laboratories (Kansas City, Mo.); mixed bacterial vaccine (MBV = heat-killed *Streptococcus pyogenes* and *Serratia marcescens*) from Bayer Co. (Wuppertal, Germany); *Brucella abortus* vaccine from the U.S. Department of Agriculture (Ames, Iowa); and poly(I)-poly(C) from P-L Biochemicals (Milwaukee, Wisc.).

RESULTS

Assay for tumor necrosis factor (TNF) in serum

The criterion adopted as a standard for assaying TNF in the sera of mice subjected to various treatments (see below) was visual observation of necrosis in a subcutaneous transplant of BALB/c sarcoma Meth A. Fig. 1 illustrates the grades of re-

sponse (- to +++) elicited in individual (BALB/c × C57BL/6)F₁ mice by administration of serum containing TNF. In the maximum (+++) response, the major part of the tumor mass is destroyed, leaving only a peripheral rim of apparently viable tumor tissue. In about 25% of mice treated with 0.5 ml of TNF-positive serum, the tumor regresses. Regression is not seen in control untreated mice under these conditions. Mice receiving TNF-positive serum show no marked signs of toxicity.

Necessity for treatment with both BCG and endotoxin for the production of TNF in the serum

In the studies summarized in Table 1, TNF was demonstrable in the serum of BCG-infected mice given endotoxin, but not in the serum of mice given either BCG alone or endotoxin only.

Conditions for optimal production of TNF

Dose of BCG. An inoculum of 2×10^7 viable organisms was chosen because we found that this gives maximal reticulo-endothelial system (RES) stimulation and sensitization to endotoxin lethality (6).

Interval Between BCG Infection and Administration of Endotoxin. We chose an interval of 14-21 days because this is the time of maximal RES stimulation, as judged by enlargement of the spleen and liver and by phagocytic indices.

Table 1. Necrosis of sarcoma Meth A* produced *in vivo* by serum from BCG-infected CD-1 Swiss mice treated with endotoxin

Serum [†] from mice treated with:		TNF assay: Necrotic response [‡]			
		+++	++	+	—
BCG§	Endo- toxin ¶	Number of mice			
—	—				9
+	—			2	7
—	+				9
+	+	171	109		

* 7-day subcutaneous transplants of BALB/c sarcoma Meth A in (BALB/c × C57BL/6)F₁ mice; initial inoculum 2×10^5 cells; approximate diameter of tumor mass at time of assay, 7-8 mm.

† Pooled sera from female CD-1 Swiss donors; 0.5 ml iv per tumor-bearing recipient.

‡ For scoring of the necrotic response, see Fig. 1.

§ Viable organisms (2×10^7) iv per mouse 14 days before exsanguination.

¶ Twenty-five micrograms iv per mouse 2 hr before exsanguination.

Abbreviations: TNF, tumor necrosis factor; BCG, bacillus Calmette-Guérin; iv, intravenous; RES, reticulo-endothelial system; MEF, mouse embryo fibroblasts.

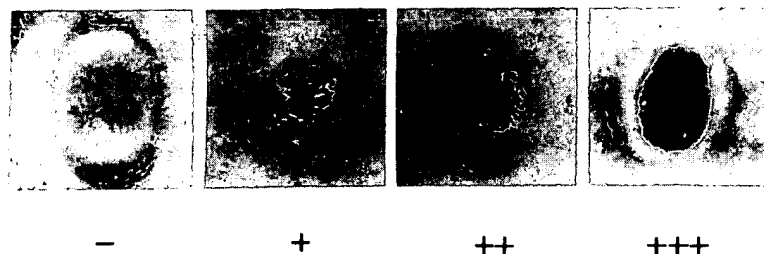


FIG. 1. Grading of the necrotic responses of BALB/c sarcoma Meth A (7-day subcutaneous transplants) 24 hr after administration of 0.5 ml of TNF-positive serum intravenously (iv). Histology shows corresponding devastation of tumor cells; hemorrhage is only a minor feature at this stage.

Dose of Endotoxin. In the range of 0.25–25 μ g per mouse, the highest dose was most consistently effective in eliciting TNF in BCG-infected mice.

Time of Serum Collection. Intervals of 30 min to 4 hr after endotoxin (in BCG-infected mice) were investigated. The optimal time for collecting serum was found to be 2 hr. Although at this time the mice were acutely shocked, their blood volume was still sufficient for a good yield, whereas later they were moribund with circulatory collapse. (Clotting of blood from donors yielding TNF is minimal or absent; in fact poor clotting is a good indication that a particular serum will show TNF activity. We refer to the supernate obtained after centrifugation of pooled blood as "serum," although its composition may be closer to plasma.)

Conditions for optimal assay of TNF

(a) The BALB/c ascites sarcoma Meth A (inoculated subcutaneously in counted numbers) was chosen for the standard TNF assay because we knew it to be susceptible to "hemorrhagic necrosis" produced by endotoxin, and because we are familiar with its highly consistent growth characteristics. Although the assay can be conducted equally effectively with Meth A in syngeneic BALB/c hosts, the (BALB/c \times C57BL/6) F_1 hybrid was preferred because these mice were available in greater numbers. (b) As in the case of endotoxin itself, TNF produced its most consistent effect on well-established (7-day) transplants, less effect on 6-day transplants and virtually none on 5-day transplants. (c) In the standard assay, the necrotic response generally corresponds with the volume of TNF-positive serum administered in the range

0.1 ml (usually negative)–0.5 ml (+++ reaction). (d) We found 24 hr to be the optimal time to score TNF reactions. Reactions are already evident after 3–4 hr.

Other priming and eliciting agents

As Table 2 shows, *C. granulorum*, *C. parvum*, and Zymosan are as effective as BCG as priming agents for TNF release by endotoxin. These agents have in common their capacity to produce marked RES hyperplasia. Two agents in addition to endotoxin, mixed bacterial vaccine and poly(I)-poly(C), elicited TNF in BCG-primed mice, whereas old tuberculin and *B. abortus* did not.

Estimation of residual endotoxin in TNF-positive sera

Two assays were used to detect residual endotoxin in TNF-positive sera. In the standard rabbit pyrogenicity assay (7), the two separate pools of TNF-positive sera tested showed endotoxin levels of ≤ 0.01 μ g/ml and 0.022 μ g/ml. Sera from normal mice, endotoxin-treated mice, or BCG-treated mice (Table 1) were non-pyrogenic. In the *Limulus* assay (8), endotoxin levels estimated at 1 μ g/ml were found in the serum of endotoxin-treated mice, whether pretreated with BCG or not. These amounts of residual endotoxin in TNF-positive sera are less than 0.1–1% of the amount necessary to produce comparable hemorrhagic necrosis in Meth A.

Effect of TNF on other tumors

A high degree of sensitivity to TNF, comparable to that of Meth A, was observed with the following transplanted tumors, growing subcutaneously in the indicated mouse strains: sarcomas S-180 (CD-1 Swiss) and BP8 (C3H); leuke-

Table 2. Assays for TNF in the serum of mice treated with various priming and eliciting agents

Treatment of serum donors*		TNF assay: Necrotic response†			
Priming agent	Eliciting agent‡	+++	++	+	—
		Number of mice			
BCG (2×10^7 viable organisms)	Endotoxin (25 μ g)	171	109		
	Mixed bacterial vaccine (5 μ l)	10	6	3	
	poly(I)-poly(C) (200 μ g)		2		9
	Old tuberculin (50 μ g)				9
	<i>B. abortus</i> (1×10^8 killed organisms)				11
<i>C. granulorum</i> (700 μ g) <i>C. parvum</i> (1000 μ g) Zymosan (2000 μ g)	BCG (1×10^8 viable organisms)			2	9
	Endotoxin (25 μ g)	8	7		
		24			
		4			

* CD-1 Swiss mice received the eliciting agent 14 days after the priming agent (both iv) and were exsanguinated 2 hr later.

† For scoring of the necrotic response, see Fig. 1 and Table 1.

‡ Doses are given in parentheses.

Table 3. Mouse strains compared for their capacity to produce TNF

Strain of serum donors	Treatment of serum donors *							
	BCG (2 × 10 ⁷ viable organisms) + endotoxin (25 μg)				<i>C. granulorum</i> (700 μg) + endo- toxin (25 μg)			
	TNF assay: Necrotic response †							
	+++	++	+	—	+++	++	+	—
Number of mice								
CD-1 Swiss	171	109			8			
C57BL/6	4				4			
SJL/J	3			1	4			
AKR/J	4				3	1		
A/J				4		3		1

* Donor mice received endotoxin 14 days after BCG or *C. granulorum* (both iv) and were exsanguinated 2 hr later.

† For scoring of the necrotic response, see Fig. 1 and Table 1.

mias EL4 (C57BL/6), ASL1 (A strain), RADA1 (A strain), RL31 (BALB/c), and EARAD1 (C57BL/6 \times A)F₁; and mastocytoma P815 (DBA/2). The reticulum-cell sarcoma RCS5 (SJL), which disseminates widely, was resistant. Among primary spontaneous neoplasms, AKR leukemias show intermediate sensitivity (indicated by reduction in the size of spleen and lymph nodes), and mammary tumors of (C3H/An \times I)F₁ origin were only slightly responsive. Meth A growing in the ascites form was highly sensitive to TNF given intraperitoneally, even in recipients with advanced disease.

Production of TNF by different mouse strains (Table 3)

Strains C57BL/6, SJL/J, and AKR/J produced TNF roughly as well as Swiss mice. A/J mice failed to produce TNF when primed with BCG but did so to some extent when primed with *C. granulorum*.

TNF production in other mammals (Table 4)

Both rats and rabbits produced TNF; as in the mouse, both BCG and endotoxin were required to induce appreciable amounts of TNF. Rabbits are particularly sensitive to endotoxin, so the dose was adjusted accordingly. The serum of

BCG-infected rabbits that died less than an hour after endotoxin showed little or no TNF activity.

Activity of TNF against cells in culture (Fig. 2)

This was tested with Meth A cells, L cells (NCTC Clone 929), and mouse embryo fibroblasts (MEF). The L cells proved most sensitive, Meth A sarcoma cells somewhat less so, and MEF virtually insensitive. The criterion employed was the count of viable cells after 48-hr exposure. Judging by proportional viability counts (trypan blue exclusion or phase microscopy), the effect of TNF on Meth A appears primarily cytostatic, whereas L cells die within the 48-hr test period. The toxicity is delayed; no effect of TNF is demonstrable in the first 16 hr of exposure. Measurable toxicity for L cells was demonstrable with dilutions of TNF-positive serum as high as 1:10⁴. Toxicity was not abolished by heating the TNF serum to 56° for 30 min. Sera from normal mice, or mice treated with either BCG or endotoxin alone, tested under the same conditions as TNF-active sera, showed no toxicity. Endotoxin itself, in concentrations as high as 500 μ g/ml, was not toxic for L cells. Rabbit and rat TNF sera had the same pattern of toxicity as mouse TNF, being highly toxic for L cells but not for MEF.

Correlation of toxic effects of TNF-positive sera *in vitro* and *in vivo*

In a broad range of tests, there has been no discrepancy between the TNF activity of sera against Meth A *in vivo* and their toxicity for L cells *in vitro*.

DISCUSSION

The inhibitory effect of bacterial products on human cancer has long been known (9). The counterpart in laboratory animals has been regarded as the "hemorrhagic necrosis" of transplanted tumors caused by material from Gram-negative bacteria. Much early work culminated in Shear's isolation of a tumor-necrosing "polysaccharide" (10), now recognized as endotoxin. The fact that endotoxin does not kill tumor cells in culture indicated that its action must be indirect, and lent credence to Algire's conclusion (11) that hemorrhagic necrosis might be secondary to endotoxin-induced hypotension leading to circulatory stasis and ischemia in the tumor. The discovery of TNF provides a more obvious rationale for the indirect action of endotoxin, namely, that endo-

Table 4. TNF release in rats and rabbits

Treatment of serum donors*		Serum donors							
		CD rat				NZW rabbit			
		TNF assay: Necrotic response†							
		+++	++	+	—	+++	++	+	—
		Number of mice							
BCG	Endotoxin								
—	—				8			2	6
+	—				8			1	7
—	+			1	7			1	7
+	+	5	6	1	2	11‡	23‡	10‡	7‡

* BCG (7×10^7 viable organisms iv per rat or 3×10^8 iv per rabbit) was given 14 days before endotoxin. Endotoxin (250 μ g iv per rat or 100 μ g iv per rabbit) was given 2 hr before exsanguination.

† For scoring of Meth A necrotic response, see Fig. 1 and Table 1.

‡ Heat-inactivated sera from 17 different rabbits assayed in three mice each, making a total of 51 assay mice. Each mouse was injected with 0.5 ml of rat serum or 1 ml of rabbit serum.

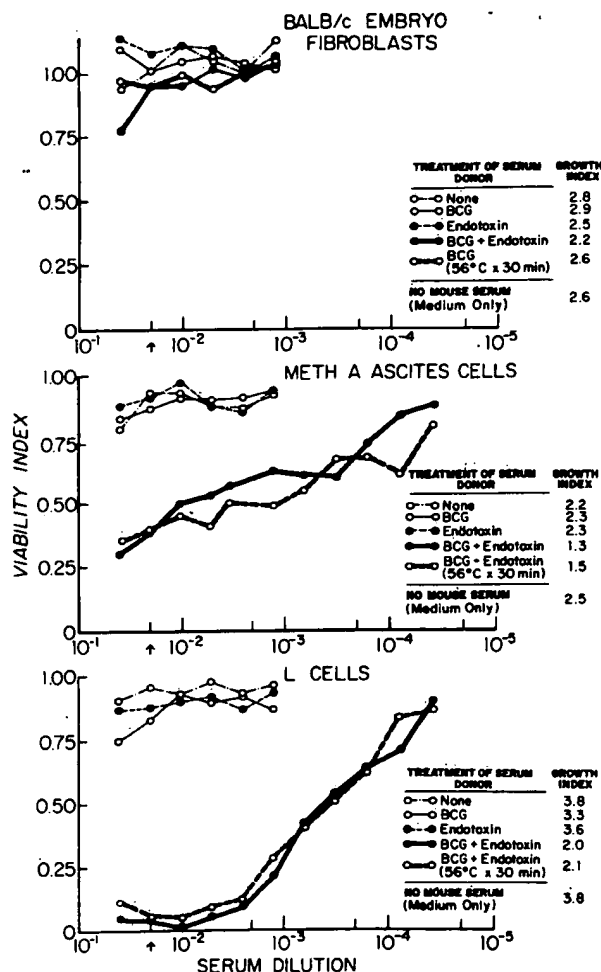


FIG. 2. Inhibition of growth of cultured cell lines by TNF-positive serum. L cells (NCTC Clone 929) and BALB/c embryo fibroblasts (MEF) were grown as monolayers, and Meth A cells in suspension. Culture medium: Eagle's minimum essential medium plus nonessential amino acids, 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). One milliliter of cell suspension (2×10^5 MEF or L cells per ml; 2.5×10^4 Meth A cells per ml) was plated with 1 ml of the mouse serum to be tested, in serial dilution (abscissa), or 1 ml of culture medium (standard). Incubation: 48 hr in 5% CO_2 in air at 37°. Viability index = number of viable cells present in culture with test serum, divided by number in culture with medium alone. Insets: growth index = total number of cells (viable and dead) after 48 hr in culture in 1/50 mouse serum (f) divided by number of cells plated. (Replicate plates; duplicate readings.)

toxin causes the host to release a factor which is toxic for the tumor. The conclusion that TNF found in the serum mediates tumor necrosis produced by endotoxin is further supported by the fact that both agents, TNF and endotoxin, act against a similar spectrum of tumors and at a similar phase of their growth.

Partial characterization of TNF indicates a glycoprotein with a molecular weight of about 150,000 which migrates with α -globulins (12). This glycoprotein has the characteristic properties of TNF-positive serum, i.e., necrosis of Meth A *in vivo* and toxicity for L cells *in vitro*. It does not contain the sugar 2-keto-3-deoxyoctonate (KDO) or the 3-D-myristoyl fatty acid characteristic of the Lipid A moiety of endotoxin from *E. coli* (13). Can TNF be identified with

any of the factors already known to be elicited by endotoxin? So far we can exclude interferon, which, although abundant in TNF sera, is absent from partially purified TNF. C-reactive protein has been ruled out, and the stability of TNF at 56° excludes any heat-labile factor.

The cellular origin of TNF is uncertain, but the fact that macrophage-inciting agents are necessary for its demonstration in serum points to this cell as the source. This interpretation is strongly supported by recent observations we have made on TNF-donor mice (S. Sternberg, unpublished data). In the greatly enlarged spleens of BCG-infected mice, microscopy shows massive hyperplasia of macrophages; two hours after administration of endotoxin, at the time when TNF is abundant in the blood, there is pyknosis and disruption of this cell population.

One of the most provocative findings about macrophages in recent years is that when nonspecifically "activated" by agents such as BCG, endotoxin, and certain protozoa, they acquire selective toxicity for malignant cells (14-17). The fact that TNF *in vitro* showed discriminatory toxicity for transformed cells in our present study might suggest that TNF mediates the selective cytotoxicity of activated macrophages.

To explore the full potential of TNF, a more plentiful source will be required. Extraction from liver and spleen of mice with RES hyperplasia produced by BCG or *C. parvum* is one possibility; but the fact that the rat and rabbit produce TNF suggests the use of larger animals for preparing TNF in quantity.

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Spatial and temporal control of gene therapy using ionizing radiation

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Activation of transcription of the *Egr-1* gene by X-rays is regulated by the promoter region of this gene. We linked the radiation-inducible promoter region of the *Egr-1* gene to the gene encoding the radiosensitizing and tumoricidal cytokine, tumour necrosis factor- α (TNF- α) and used a replication-deficient adenovirus to deliver the Egr-TNF construct to human tumours growing in nude mice. Combined treatment with Ad5.Egr-TNF and 5,000 cGy (rad) resulted in increased intratumoral TNF- α production and increased tumour control compared with treatment with Ad5.Egr-TNF alone or with radiation alone. The increase in tumour control was achieved without an increase in normal tissue damage when compared to tissue injury from radiation alone. Control of gene transcription by ionizing radiation *in vivo* represents a novel method of spatial and temporal regulation of gene-based medical treatments.

The potential of gene therapy in the treatment of cancer is the delivery of therapeutic agents to tumour cells to alter the malignant phenotype or to induce tumour cell cytotoxicity^{1,2}. Gene therapy strategies currently under clinical investigation for the treatment of cancer include the study of genes that activate or encode cytotoxins and immune modulators^{3,4}. These strategies are frequently limited by inherent drug resistance of tumour cells or by poor diffusion of the gene product. The cytotoxic proteins, such as ricin and pseudomonas endotoxin, must also be restricted to the local tumour site to avoid potential systemic toxicity. Methods used for localizing cytotoxic gene therapy for cancer include viral delivery systems that take advantage of tissue-specific receptors⁵ and tissue-specific enhancers that limit transcription to certain cell types⁶. However, despite these advances, localizing gene therapy to the tumour remains a major obstacle. Furthermore, the temporal regulation of cytotoxic genes is not possible with constitutive promoters that have a high level of basal gene expression. Alternatively, transcriptional activation of a radiation-inducible promoter can be controlled by ionizing radiation within a specific volume and for a chosen period. We have taken advantage of both the killing effect and the targeting potential of ionizing radiation to achieve spatial and temporal regulation of TNF- α gene transcription and enhance tumour cell killing.

Tumour necrosis factor- α (TNF- α) is a cytokine that activates the cellular immune response⁷ and is directly cytotoxic to some tumour cells⁸. Mechanisms of direct cell killing by TNF- α involve both apoptosis and necrosis^{9,10}. When combined with radiation *in vivo*, TNF- α is reported to enhance tumour control through immune modulation¹¹. We and others have reported that TNF- α

enhances direct tumour cell killing *in vivo* and *in vitro* following exposure to ionizing radiation¹²⁻¹⁷ and a clinical study that combined systemically (intravenously) administered TNF- α and therapeutic local/regional radiation demonstrated promising results in local tumour control¹⁸. In that study, increased serum concentrations of TNF- α correlated with an increase in local control in the irradiated tumour bed. However, systemic toxicity attributable to TNF- α limited the therapeutic efficacy of this treatment regimen. We propose that a gene therapy approach combining high intratumoral TNF- α levels induced by regional radiation exposure might limit systemic toxicity while achieving local tumour control.

Although numerous radiation-inducible genes^{19,21}, and proteins²² have been identified, relatively few radiation inducible promoters/enhancers have been characterized^{23,24}. Promoter/enhancers are DNA sequences that bind proteins which control gene transcription. DNA sequences that activate transcription after X-irradiation include AP-1 (ref. 23), the NF κ B binding sequence²⁵ and the CC(A+T rich)₆GG (CAR_G) elements within the 5' untranslated region of the early growth response (*Egr-1*) promoter²⁴. We selected the CAR_G elements of the *Egr-1* promoter (425 bp upstream from the transcription start site) to regulate TNF- α , because these elements are inducible in several types of human tumour cells^{24,26} (D.E.H., unpublished observation). We ligated a region containing the six CAR_G elements of the promoter/enhancer region of the *Egr-1* gene upstream to a TNF- α cDNA. The replication-deficient adenovirus type 5 (Ad5)^{27,28} was used to deliver the Egr-TNF genetic construct to tumours. The Ad5.Egr-TNF vector was injected into the human epithelial tumour (SQ-20B) xenograft. SQ-20B is a radioresistant tumour cell

line derived from a human laryngeal carcinoma^{29,30}. The schedule of delivery of Ad5.Egr-TNF and radiation was chosen to simulate treatment in the clinical setting. We show that the decrease in tumour volume after administration of Ad5.Egr-TNF and radiation is associated with TNF- α induction and an increase in apoptosis, as well as in necrosis and inflammation. These effects were not observed using the control Ad5(null) virus combined with radiation.

Ad5.Egr-TNF and therapeutic radiation

To study a potential interaction between Ad5.Egr-TNF and radiation, SQ-20B xenografts were grown to a mean volume of 160 mm³ and injected with Ad5.Egr-TNF. Control tumours were uninjected (Fig. 1a). Ad5.Egr-TNF alone or radiation alone produced tumour regression to a mean of 30% (day 28) or 51% (day 25) of the original volume, respectively (Fig. 1a). Tumour regression in these groups of animals was followed by tumour regrowth to the original volume by day 42 and 38 post-treatment (Fig. 1a). In the group of mice treated with Ad5.Egr-TNF plus radiation, the mean tumour volume was reduced to 16% of the original volume at day 38 ($P < 0.05$) with no tumour regrowth. At day 60, Ad5.Egr-TNF combined with X-irradiation produced a 90% reduction in tumour volume in 12 of 16 tumours, compared with radiation alone (10 of 23 tumours), or Ad5.Egr-TNF alone (5 of 16 tumours; $P < 0.04$, ANOVA).

Tumours were treated with the Ad5(null) virus with and without radiation. Xenografts were grown to a mean volume of 152 mm³. Tumours were injected with Ad5(null) virus twice weekly for 2 weeks, either alone or in combination with X-irradiation (5 Gy day⁻¹, 4 days per week) to a total dose of 40 Gy. Uninjected tumours were treated with a total dose of 40 Gy. We detected no effect on tumour growth of the Ad5(null) virus (1×10^8), with or without radiation (Fig. 1b). Similar results were obtained from tumours treated with Ad5(null) at 2×10^8 (data not shown). The data are consistent with our apoptosis findings *in vitro* and pathological studies, which demonstrate no interaction between null virus and radiation (see below).

Radiation cures a greater percentage of small tumours than of large tumours, which have a greater cell burden. In order to determine whether combined treatment with Ad5.Egr-TNF and radiation would be effective in controlling larger tumours, we divided tumours into 'small' and 'large' groups. The mean tumour volume of 160 mm³ was chosen to divide tumours by size. The mean volume of the large tumours was 234 ± 25 mm³ whereas the mean volume of the small tumours was 114 ± 6 mm³ (large versus small tumours, $P < 0.001$). In the combined treatment group, the mean volume of large tumours at day 0 was 218 ± 14 mm³, and the mean volume of small tumours was 96 ± 12 mm³ ($P < 0.001$). After X-ray treatment alone, both small (<160 mm³) and large (>160 mm³) tumours initially regressed. However, regrowth to initial volume occurred in the large tumours, whereas small tumours did not regrow (Fig. 2a). The combination of radiation and Ad5.Egr-TNF produced a similar volumetric reduction of both large and small tumours without tumour regrowth (Fig. 2b). These data suggest that induction of the radiosensitizing cytokine TNF- α enhances the efficacy of radiation therapy in large tumours, which cannot be controlled effectively with radiation alone.

Radiation inducibility of Egr-TNF

To study radiation induction of TNF- α , SQ-20B xenografts were grown to a mean volume of 148 mm³ in both hind limbs of nude

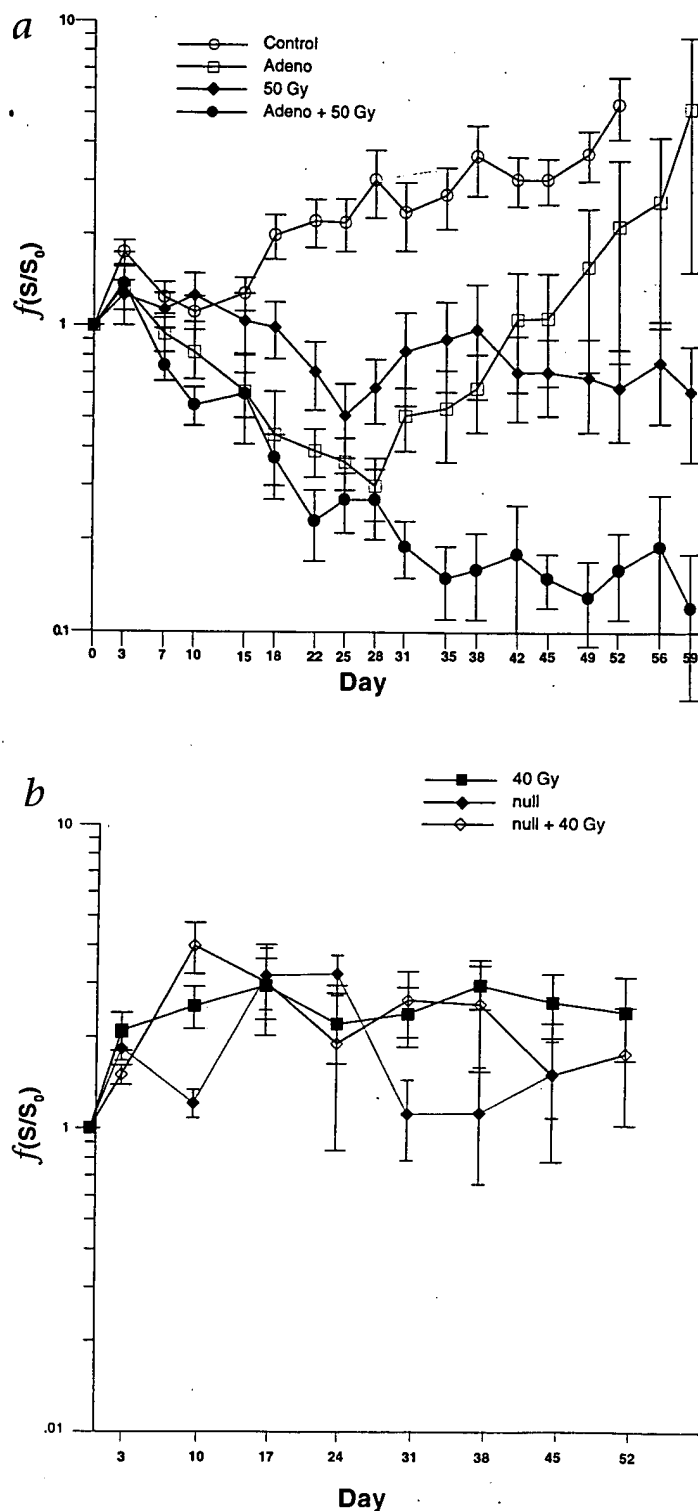


Fig. 1 Reduction of SQ-20B tumour volumes following combined treatment. SQ-20B xenografts were injected with 1×10^8 PFU of Ad5(null virus) or 2×10^8 PFU of Ad5.Egr-TNF (twice a week for two weeks). Control tumours were not injected. Tumours were irradiated (5 Gy per day, 4 days per week) to a total dose of 40 or 50 Gy. **a**, Volumes of xenografts are shown following treatment with: Control (untreated); radiation alone at 50 Gy; Ad5.Egr-TNF alone; Ad5.Egr-TNF and 50 Gy. Data are calculated as the percent of original (day 0) tumour volume and graphed as fractional tumour volume \pm s.e.m. **b**, Volumes of xenografts are shown following treatment with: 40 Gy; Ad5(null virus); Ad5 (null virus) + 40 Gy.

mice. Each tumour was injected with 2×10^8 PFU Ad5.Egr-TNF. Tumours in the right hind limbs were irradiated with 5 Gy, four times each week, to a total of 50 Gy. Unirradiated tumours in the left limbs of the same mice served as controls. TNF- α concentrations in tumour extracts were analysed using an ELISA assay. Median TNF- α concentrations in irradiated tumours were 2,846, 4,300 and 20,000 pg mg $^{-1}$ tumour protein at 7, 14 and 21 days, respectively. Unirradiated tumours had median TNF- α concentrations of 945, 950 and 2,643 pg mg $^{-1}$, respectively ($P = 0.058$, Kruskal-Wallis test)³⁴. This ELISA does not detect murine TNF- α and is specific for TNF- α produced by the human cDNA (D.E.H., unpublished observations). These data demonstrate that a single administration of the Ad5.Egr-TNF vector is sufficient for radiation induction of TNF- α protein for at least 21 days, and that TNF- α levels accumulate when repeated induction is achieved during fractionated radiotherapy.

TNF- α immunohistochemistry

To analyse the distribution of Ad5.Egr-TNF, SQ-20B tumour xenografts were grown to a mean volume of 148 mm 3 in the hind limbs of nude mice. Tumours received a single injection of 2×10^8 PFU of Ad5.Egr-TNF and were irradiated with a single 5 Gy dose. Immunohistochemical staining of tumour cryosections with goat anti-human recombinant TNF- α antibody was performed 24 hours after irradiation. Granular intracytoplasmic staining for TNF- α was found within infected tumour cells, suggesting that TNF- α was being packaged for secretion (Fig. 3a). TNF- α was released by the infected cells and diffused into the tumour interstitium at 48 hours, as indicated by pink staining on non-infected tumour cells (Fig. 3b). The finding that TNF- α diffuses to adjacent tumour cells suggests that anti-tumour activity mediated by TNF- α when combined with radiation may be due to a bystander effect. Such an effect, produced by TNF- α gene therapy, is consistent with our recent finding demonstrating that a cell-based delivery system containing Egr-TNF- α achieves interactive killing with radiation in SQ-20B tumour xenografts even though the xenograft SQ-20B cells themselves did not contain the TNF- α gene¹⁴.

Apoptosis induced in SQ-20B cells

To determine whether apoptosis contributes to tumour cell killing with the combination of radiation and TNF- α , we infected SQ-20B cells with Ad5.Egr-TNF at a multiplicity of infection (MOI) of 10, followed 24 hours later by a single dose of 20 Gy. Cells were fixed and DNA fragmentation was quantified by terminal transferase assay³¹ and fluorescence-activated cell sorting analysis. Forty-eight hours after irradiation, cells treated with either 20 Gy X-irradiation alone (Fig. 4a) or Ad5.Egr-TNF alone (Fig. 4b) demonstrated no apoptosis. In contrast, 30% of the cells receiving combined treatment of Ad5.Egr-TNF and 20 Gy demonstrated DNA fragmentation (Fig. 4c) and membrane blebbing (not shown), both characteristics of apoptosis. The correlation between TNF- α concentration and apoptosis was verified by addition of 100 pg ml $^{-1}$ human recombinant TNF- α to the culture medium 4 hours before a single dose of 20 Gy. This concentration of exogenous TNF- α produced a similar degree of apoptosis as compared with that achieved with a MOI of 10 (data not shown). Conversely, the addition of the Ad5(null) virus at a MOI of 10, followed by radiation, did not produce apoptosis. These results suggest that the combination of high levels of TNF- α and radiation results in tumour cell killing by apoptosis.

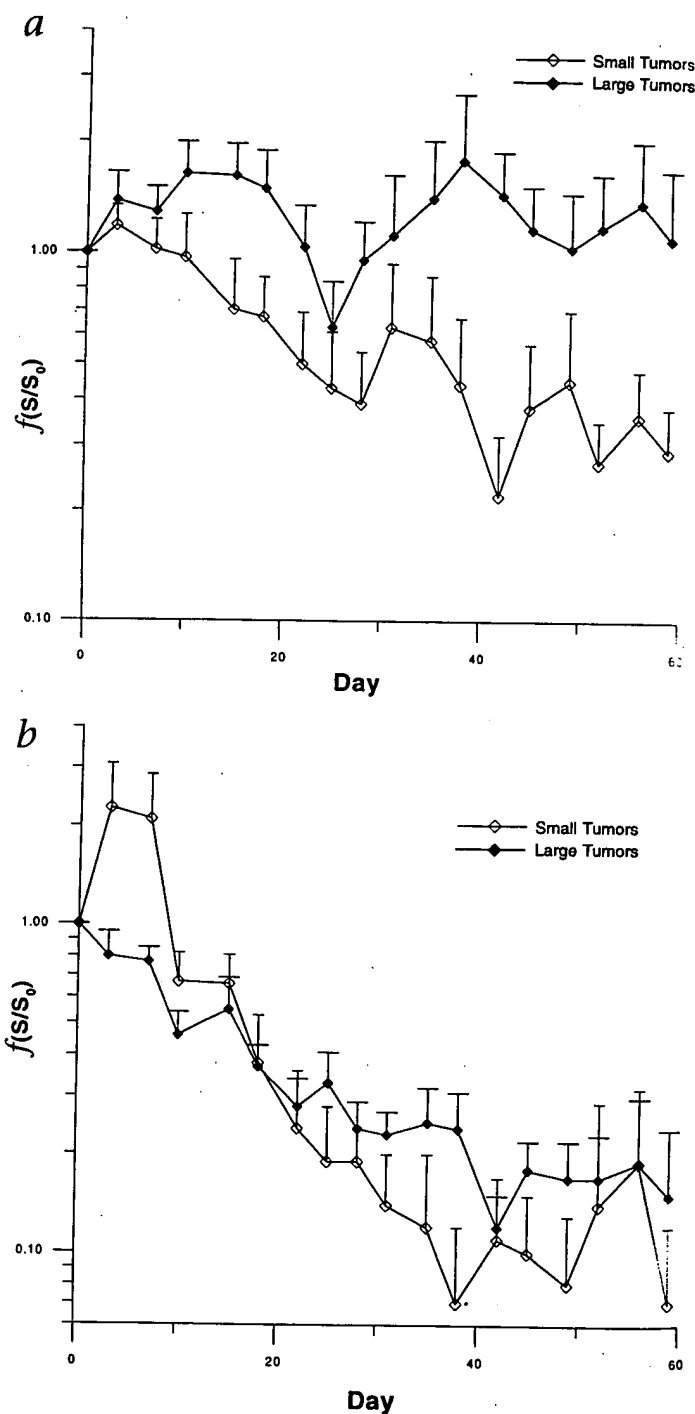


Fig. 2 Treatment effects on large versus small tumours. *a*, Mean volumes of large (>160 mm 3) and small (<160 mm 3) xenografts after treatment with radiation alone. The mean of large tumours at day 0 was 234 ± 25 mm 3 and the mean of small tumours at day 0 was 114 ± 6 mm 3 . *b*, Mean volumes of large (>160 mm 3) and small (<160 mm 3) xenografts after treatment with Ad.Egr-TNF and radiation. The mean of large tumours at day 0 was 218 ± 14 mm 3 and the mean of small tumours was 96 ± 12 mm 3 .

Necrosis in SQ-20B xenografts

To determine the extent to which necrosis is a component of tumour control, we analysed histologic sections of SQ-20B xenografts treated with two injections of Ad5(null) virus plus 20 Gy (5 Gy per day for 4 days) or with two injections of Ad.Egr-TNF

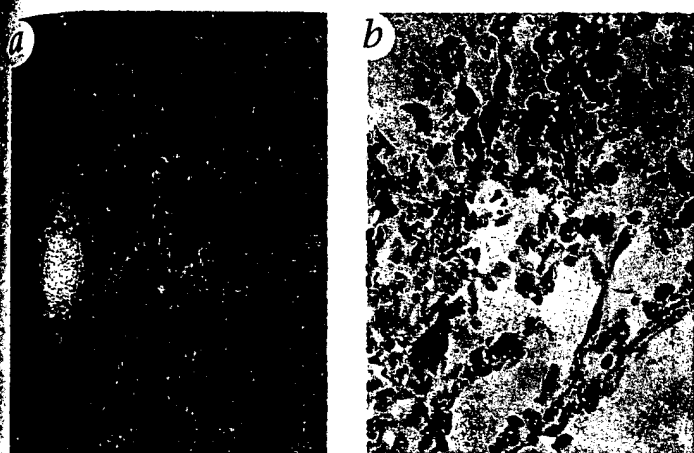


Fig. 3 TNF- α immunohistochemistry. Cryosections of the neoplasms were performed 24 hours after injection of 2×10^8 PFU Ad.Egr-TNF and 5 Gy X-irradiation. Sections were fixed in formalin and incubated with goat anti-human TNF- α monoclonal antibody followed by secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG) and stained for the presence of TNF- α protein. Slides were counterstained with haematoxylin. Shown are tumours stained at 24 hours after infection demonstrating TNF staining within granules (at $\times 1020$ magnification) (a) and tumours excised at 48 hours after treatment (b) showing TNF in the tumour interstitium ($\times 472$ magnification).

plus 20 Gy. Xenografts treated with both Ad.Egr-TNF and radiation (Fig. 5a) had necrosis over a mean of $38 \pm 8.3\%$ of the low-power fields (ten fields counted) within seven days, whereas tumours treated with Ad5(null virus) and radiation had necrosis over a mean of $1.8 \pm 0.97\%$ of the low-power fields ($P = 0.007$) (Fig. 5b). This is supported by recent findings that direct tumour cell killing by TNF- α is associated with necrosis^{7,9}.

Toxicity of Egr-TNF combined with radiation

TNF- α mediated toxicity was evaluated in mice receiving 50 Gy and Ad.Egr-TNF. Serum levels of TNF- α were quantified by ELISA. No human TNF- α protein could be detected (<8 pg ml⁻¹) and there was no weight difference between animals treated with Ad5.Egr-TNF and X-irradiation and those receiving either treatment alone. No animals died in any of the treatment groups throughout the course of the experiment. In spite of mild local oedema and fibrosis, no loss of hind-limb mobility and no skin desquamation were observed in groups of animals receiving radiation and Ad.Egr-TNF. The soft tissue toxicity of Ad.Egr-TNF and radiation was comparable to that observed in animals receiving radiation alone. The minimal local effects of the TNF- α /radiation combination are similar to the effects observed in a clinical trial combining systemic recombinant TNF- α and radiotherapy¹⁸. The present data suggest that TNF- α production is localized to the tumour bed resulting in no systemic and only minimal local toxicity. Studies of possible long-term effects of TNF- α /radiation compared with radiation alone are under way.

Discussion

We have used TNF- α as a therapeutic gene for radiation-regulated gene therapy because of its direct anti-tumour effect and its interactive killing when combined with ionizing radiation. We observed that production of TNF- α in human tumour xenografts infected with the Ad.Egr-TNF and treated with radiation enhances

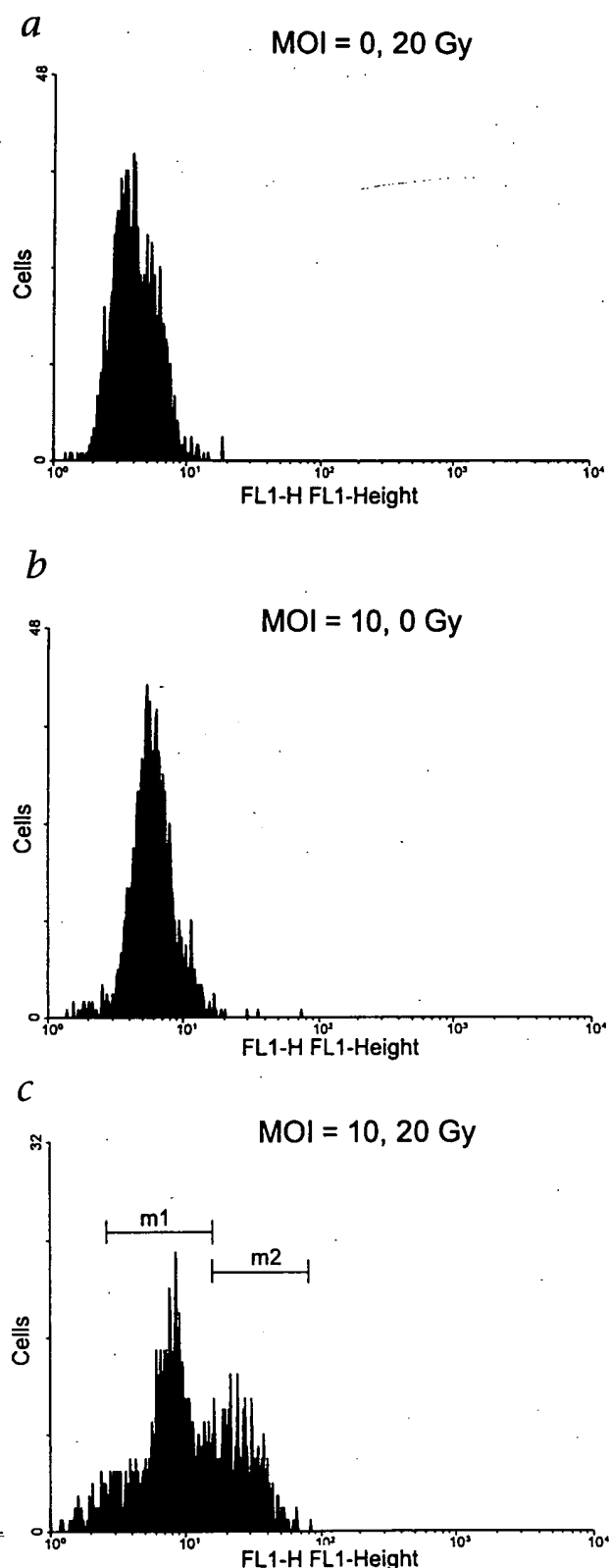


Fig. 4 Apoptosis of SQ-20B cells *in vitro*. SQ-20B tumour cells were treated with Ad.Egr-TNF at an MOI of 10, followed by 20 Gy, 24 hours later. At 48 hours after irradiation the presence of DNA strand breaks was evaluated. Analysed regions (gated regions) were set to quantify the number of cells undergoing DNA fragmentation. a, Cells treated with 20 Gy alone. b, Cells treated with Ad.Egr-TNF alone (MOI 10). c, Cells treated with 20 Gy and Ad.Egr-TNF (MOI 10).

tumour control as compared with radiation alone. These data are supported by *in vitro* studies demonstrating that TNF- α enhances tumour cell killing by radiation¹³ and *in vivo* studies showing improved tumour control^{11,16,17}. Unfortunately, a clinical trial combining TNF- α and radiation was limited by systemic toxicity¹⁸. The present work suggests that localized production of TNF- α may enhance tumour killing while avoiding systemic toxicity.

Interactive killing between TNF- α and radiation was associated with both apoptosis and necrosis. Although apoptosis has been associated with direct tumour cell killing by TNF- α (ref. 9) and radiation³², we found that neither Ad.Egr-TNF, nor radiation alone resulted in apoptosis in the SQ-20B tumour cell line, whereas only cells receiving both TNF- α and radiation demonstrated both DNA fragmentation and membrane blebbing, characteristics of apoptosis. The apoptosis that we observed in this study was reproduced by treating SQ-20B tumour cells *in vitro* with recombinant TNF- α and radiation, but not by the combination of null virus (Ad5) and radiation, confirming that the effect was due to TNF- α rather than the vector itself. The apoptosis observed with concomitant Ad.Egr-TNF and radiation may have clinical significance, because Meyn *et al.* showed that murine tumours exhibiting a large apoptotic fraction following irradiation were more likely to be brought under control³².

The inflammatory infiltrate seen in Fig. 5a suggests a further possible contribution of immune stimulation by TNF- α . The inflammatory component is consistent with findings observed with recombinant TNF- α combined with radiation *in vivo*¹¹. Alternatively, the infiltrate may be due to the presence of the Ad5 vector itself, as this vector has been shown to induce IL-8 expression, which is a potent inducer of neutrophil infiltration³³. However, SQ-20B xenografts injected with Ad5(null) virus demonstrated 36% less infiltration by neutrophils than tumours treated with Ad.Egr-TNF, suggesting that the inflammatory component observed in tumours treated with Ad.Egr-TNF was due to the presence of TNF- α .

Gene activation targeted by ionizing radiation is a new concept for cancer treatment whereby transcription of therapeutic genes is localized and regulated by ionizing radiation. The combination of proteins produced by targeted genes with the cytotoxic effect of ionizing radiation may enhance tumour cures without a significant increase in local or systemic toxicity. We found that radiation-induction of the Egr-TNF construct resulted in a ten-fold increase in TNF- α protein levels. The induction of TNF- α protein in irradiated tumours, compared with controls at 21 days, is consistent with our *in vitro* findings demonstrating an increase in *Egr-1* expression after irradiation²⁴. Temporally fractionated radiation provides a method for repeated gene induction resulting in prolonged, accentuated gene expression. The concept of precisely activating gene transcription spatially and temporally by use of ionizing radiation may have wider applications in gene therapy and cancer treatment.

Methods

Growth of human tumour xenografts *in vivo*. We injected 10^6 SQ-20B tumour cells into the right hind limbs of nude mice. Xenografts were grown for 2–3 weeks, after which time tumours underwent spontaneous regression in less than 8% of mice. During treatment, tumour volumes were measured with calipers twice weekly and presented as per cent of original tumour volume. Tumours were grown to a mean volume of 152 mm^3 or 160 mm^3 . Xenografts were injected with 1×10^6 to 2×10^6 PFU of Ad5(null virus) or 2×10^6 PFU of Ad.Egr-TNF (2 per week for 2 weeks). Control tumours were not

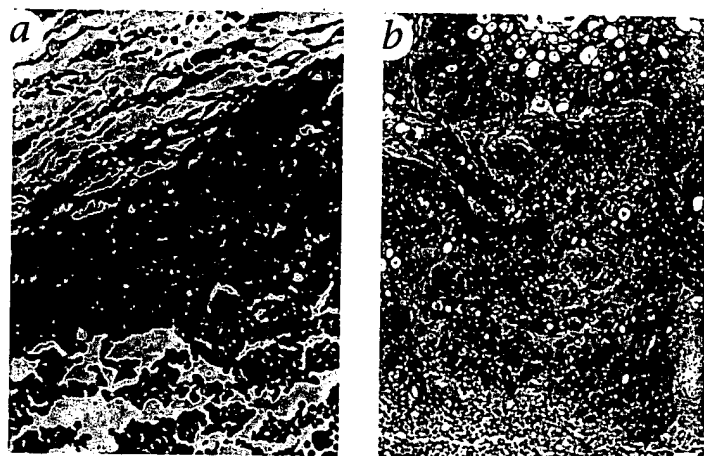


Fig. 5 Necrosis in SQ-20B xenografts receiving Ad.Egr-TNF and radiation. *a*, Tumours treated with Ad.Egr-TNF and X-irradiation (5 Gy per day for 4 days) were excised on day 7, embedded in paraffin, sectioned and stained with haematoxylin and eosin. *b*, Histologic sections of tumour treated with Ad5(null) virus and radiation (5 Gy per day for 4 days). Methods are as described as in the Methods section.

injected. Irradiated mice were immobilized in lucite chambers and the entire body was shielded with lead except for the tumour bearing hind limb¹⁴. Tumours were irradiated (5 Gy day⁻¹, 4 days per week) to a total dose of 40 or 50 Gy using a Maxitron generator (1.88 Gy min⁻¹). Tumour volumes were calculated by the formula ($a \times b \times c/2$) which was derived from the formula for an ellipsoid ($\pi d^3/6$). Both the Ad5(null virus) and the Ad.Egr-TNF vector (GenVec, Rockville, Maryland) were recombined with a replication-deficient adenovirus type 5 (ref. 27). Tumours were treated with radiation alone; Ad5(null virus) alone; Ad.Egr-TNF alone; Ad5(null virus) combined with radiation; or Ad.Egr-TNF combined with radiation. Data were calculated as the percent of original (day 0) tumour volume and graphed as fractional tumour volume \pm s.e.m. The regression rate of large ($>160 \text{ mm}^3$) xenografts was compared with small ($<160 \text{ mm}^3$) xenografts following treatment with radiation alone or Ad.Egr-TNF plus radiation. This tumour volume was selected because it represents the mean volume of the tumours treated with TNF- α .

TNF- α expression in xenografts. SQ-20B xenografts were grown to a mean volume of 148 mm^3 in both hind limbs of nude mice. Each tumour was injected once with 2×10^6 PFU Ad.Egr-TNF 4 hours before the first dose of radiation. Tumours in the right hind limbs were irradiated with 5 Gy, 4 times each week to a total of 50 Gy. Unirradiated tumours in the left limbs of the same mice served as controls. Tumours were excised, placed in tubes and frozen in liquid nitrogen. Tumours were then homogenized in 500 μ l sodium chloride/Tris buffer, pH 7.5, containing EDTA, dithiothreitol, and protease inhibitors. Homogenization on ice was for 30 s using a Brinkman Polytron and was followed by four cycles of freezing/thawing. Samples were centrifuged for 5 min at 10,000g and the supernatant assayed for TNF- α using a Quantikine TNF- α ELISA kit (R&D Systems, Minneapolis, Minnesota). Protein content was determined using the Bio-Rad Protein Micro Assay (BioRad, Melville, New York). The Kruskal-Wallis test was used for statistical analysis because it was determined that the data were not distributed normally³⁴.

TNF- α immunohistochemistry. Tumours were excised and frozen 24 hours after a single injection of 2×10^6 PFU Ad.Egr-TNF and 5 Gy X-irradiation. Cryosections of the neoplasms were performed and

tissue was fixed in formalin and incubated with goat anti-human TNF- α monoclonal antibody or normal goat serum at 1:200 dilution in PBS. Sections were then incubated with secondary antibody (horseradish peroxidase-conjugated rabbit anti-goat IgG) and stained for the presence of TNF- α protein, using Vector ABC reagent and diaminobenzidine (DAB) (Vector Labs, Burlingame, California). Slides were counterstained with haematoxylin and dehydrated, and cover slips were positioned before light microscopic evaluation.

Histology of SQ-20B xenografts. Tumours infected twice with Ad.Egr-TNF or Ad5(null virus) and treated with X-irradiation (5 Gy per day for 4 days), were excised on day 7 and fixed in 10% neutral buffered formalin. Tumours were then trimmed and processed in a Tissue Tek II Tissue Processor, embedded in paraffin, sectioned and stained with haematoxylin and eosin, and examined for signs of necrosis by light microscopy.

Terminal transferase quantification of apoptosis. SQ-20B tumour cells were treated with Ad.Egr-TNF at a MOI of 10, followed by 20 Gy 24 hours later. At 48 hours after irradiation, the presence of DNA breaks was evaluated by a modification of the nick-end translation method for *in situ* labelling of DNA breaks. Cells were fixed with 1% buffered methanol-free formaldehyde for 15 min on ice and stored in 70% ethanol overnight at -20 °C, rehydrated by washing twice in PBS and incubated in TDT buffer (25 mM Tris-HCl, 200 mM potassium cacodylate, 5 mM cobalt chloride at pH 6.6), 0.3 unit per μ l terminal deoxynucleotidyl transferase and 3 nM biotin-15-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) at 37 °C for 1 hour. The reaction was stopped by addition of ice-cold PBS and the cells were incubated for 30 min at room temperature in the dark in a solution containing 4 \times SSC buffer (Sigma), 5 μ g ml⁻¹ avidin-fluorescein-isothiocyanate (Boehringer Mannheim), 0.1% Triton X-100 (vol/vol), and 0.5% nonfat milk. The reaction mixture was washed once in ice-cold PBS containing 0.1% Triton X-100 (vol/vol), and cells were suspended at a concentration of 10⁴ cells per ml in PBS containing propidium iodide (5 μ g ml⁻¹) and 0.1% RNase (for 30 min at room temperature). The red (propidium iodide) and green (fluorescein) fluorescence was measured with a FACSCAN flow cytometer (Becton Dickinson, San Jose, California), and the data were analysed with LYSYS II software (Hewlett Packard, Palo Alto, California). Analysis regions (gated regions) were set to quantify the number of cells undergoing DNA fragmentation. Cells were treated with 20 Gy alone, Ad.Egr-TNF alone (MOI 10) or 20 Gy and Ad.Egr-TNF (MOI 10).

Acknowledgements

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Tumor Necrosis Factors: Gene Structure and Biological Activities

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Tumor necrosis factor (TNF) is the name given to a serum-derived factor that is cytotoxic for many transformed cell lines in vitro and causes the necrosis of certain tumors in vivo (Carswell et al. 1975). The name lymphotoxin was proposed in 1968 for a factor with similar biological properties that is synthesized by mitogen-stimulated lymphocytes (Granger and Kolb 1968; Ruddle and Waksman 1968). Both of these activities are now known to correspond to distinct proteins. Many other proteins with cytotoxic activities have been described and given a variety of names; however, whether any of these activities can be attributed to cytokines distinct from TNF and lymphotoxin is still not clear. On the basis of structural homology and similarity in biological function, the names TNF- α and TNF- β have been given to TNF and lymphotoxin, respectively. Reviews on the current status of research on TNF- α (Old 1985; Pennica and Goeddel 1986) and TNF- β (Gray 1986) have appeared recently.

TNF Protein and cDNA Structure

Both TNF- α and TNF- β are assayed by measuring cytotoxic activity on actinomycin-D-treated L-M cells, a sensitive clone of murine L929 fibroblasts (Kramer and Carver 1986). The biochemical characterization of the TNFs was greatly aided by the identification of cell lines capable of producing these cytokines after exposure to phorbol myristate acetate (PMA). TNF- α and TNF- β were found to be produced by the human promyelocytic cell line HL-60 (Pennica et al. 1984) and the human lymphoblastoid cell line RPMI 1788 (Aggarwal et al. 1984), respectively. Both proteins were purified to homogeneity by Aggarwal et al. (1984, 1985b,c). Amino acid sequence analysis of the purified cytokines permitted the design of synthetic DNA hybridization probes that were used to screen the appropriate cDNA libraries. Cloned cDNAs corresponding to human TNF- α (Pennica et al. 1984) and TNF- β (Gray et al. 1984) were isolated and characterized.

On the basis of sequence analysis of cloned cDNAs, human TNF- α mRNA was shown to encode a precursor protein of 233 amino acids (Pennica et al. 1984). Amino-terminal sequence analysis of natural TNF- α

(Aggarwal et al. 1985c) demonstrated that the mature protein of 157 amino acids is preceded by a 76-amino-acid signal sequence involved in protein secretion. The calculated monomeric molecular weight of 17,356 agrees with the value determined experimentally by SDS-PAGE under reducing conditions. The two cysteine residues of the mature TNF- α are linked by a disulfide bridge that is essential for cytotoxic activity (Aggarwal et al. 1985c).

TNF- β mRNA contains an open reading frame of 205 codons, the first 34 of which constitute a secretion signal sequence. Native TNF- β isolated from the RPMI 1788 cell line is a glycoprotein that exists in two forms (20 kD, 148 amino acids, and 25 kD, 171 amino acids) differing by 23 amino acids at their amino termini (Aggarwal et al. 1985b). Unlike TNF- α , TNF- β does not contain any cysteine residues.

A comparison of TNF- α and TNF- β reveals a high degree of amino acid sequence homology. If two gaps are introduced, the sequences can be aligned so that 44 amino acids (28%) occur in identical positions (Fig. 1). The introduction of two additional gaps permits the alignment of nine more amino acids (34% overall homology; Aggarwal et al. 1985c). It is likely that the two highly conserved regions (amino acids 35-66 and 110-133; TNF- α numbering) are important for the similar cytotoxic activities of the two molecules and/or the recognition of the TNF receptor. Interesting differences between the two proteins are found in the amino-terminal portion and in the region from amino acids 67 to 109 (TNF- α numbering), where there are only two identical residues.

Expression plasmids were constructed that direct the synthesis in *Escherichia coli* of mature recombinant human TNF- α (Pennica et al. 1984) and TNF- β (Gray et al. 1984). Both recombinant products have been purified to homogeneity free of contaminating lipopolysaccharide (LPS or endotoxin). Specific activities of approximately 10^8 U/mg were determined for both TNFs in the L-M cell in vitro cytotoxic assay.

The amino acid sequences of TNF- α (murine, rabbit, and bovine) and TNF- β (murine and bovine) from other species have been deduced by cDNA and genomic DNA sequencing. These amino acid sequences are compared in Figures 2 and 3. The high degree of amino acid con-

Figure 1. Comparison of the amino acid sequences of TNF- α and TNF- β . Two gaps have been introduced into the TNF- β sequence to increase the homology. Asterisks indicate identical amino acids.

TNF Gene Structure

distinct cell types. To understand more precisely their differential regulation, we compared the structure and organization of the human TNF genes (Nedwin et al. 1985). Human TNF- α and TNF- β were found to each be encoded by a single gene. Furthermore, both genes are about 3 kbp in size and are split by three introns. However, only the third intron is located in a homologous position in the two genes (preceding amino acids 18 and 35 of TNF- α and TNF- β , respectively; see Fig. 1). Only the fourth exons, which encode the majority

Human	-76	MSTESMIRDVELAEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTFLCLLHFGVIGPQR	
Bovine	-77	MSTKSMIRDVELAEVLSEKAGGPQGSRRCLCLSLFSFLLVAGATTFLCLLHFGVIGPQR	
Rabbit	-79	MSTESMIRDVELAEGPLPKKAGGPQGSRRCLCLSLFSFLLVAGATTFLCLLHFRVIGPQE	
Mouse	-79	MSTESMIRDVELAEALPQKMGGFQNSRRCLCLSLFSFLLVAGATTFLCLLNFVIGPQR	
Consensus		MST SMIRDVELAE L K GG Q S CL LSLFSFL VAGATTFLCLL F VIGPQ	
1			
Human	-16	EEF-PRDLSLISPLAQA--VRSSSRTPSDKPVAVHVVANPQAEGLQWLNRANALLANGV	
Bovine	-17	EEQVPSGPSINSPLVQ--TLRSSQASSNKPVAHVVDINSPGQLRWDSYANALMANGV	
Rabbit	-19	EEQSPNNHLVNPVAVMVLRSASRALSDKPLAHVVANPQVEGLQWLSQRANALLANGM	
Mouse	-19	DEKFPNGLPLISSMAQTLTLRSSQNSSDKPVAVHVVANHQVEEQLEWLSQRANALLANGM	
Consensus		E P Q RS S S KP AHVVA QL W ANAL ANG	
Human	42	ELRDNLVVPSEGLYLIYSQVLFKGGCPSTHVLLTHTISRIAVSYQTKVNLLSAIKSPC	
Bovine	42	KLEDNLVVPAGEGLYLIYSQVLFKGGCP-PPVLTHTISRIAVSYQTKVNLLSAIKSPC	
Rabbit	42	KLTDNLVVPADGLYLIYSQVLFSGGCR-SYVLLTHTVSRFAVSYPNKNVLLSAIKSPC	
Mouse	42	DLKDNLVVPADGLYLVYSQVLFKGGCP-DYVLLTHTVSRFAISYQEKVNLLSAVKSPC	
Consensus		L DNQLVVP GLYL YSQVLF GGGC LTHT SR A SY KVN LSA KSPC	
Human	102	QRETPEGAEAKPWYEPYILGGVFQLEKGDRLSAEINRPDYLDFAESGQVYFGI IAL	157
Bovine	101	HRETPEWAEAKPWYEPYIQGGVFQLEKGDRLSAEINLPDYLDYAESGQVYFGI IAL	156
Rabbit	101	HRETPEEAEPMAWYEPYILGGVFQLEKGDRLSTEVNQPEYLDLAEAGQVYFGI IAL	156
Mouse	101	PKDTPEGAELKPWYEPYILGGVFQLEKGDQLSAEVLNPKYLDFAESGQVYFGVIAL	156
Consensus		TPE AE WYEPYI YGGVFQLEKGD LS E N P YLD AESGQVYPG IAL	

Figure 2. Amino acid sequences of human (Pennica et al. 1984), bovine (D. Goeddel et al., unpubl.), rabbit (Itoh and Wallace 1985), and murine (Pennica et al. 1985) TNF- α . (-79 to -1) Amino acids of the signal sequences. "Consensus" indicates residues that are identical in all four sequences.

of the mature proteins, have significant DNA sequence homology (56%).

The human TNF genes were localized to the p23→q12 region of chromosome 6 through Southern blot analysis of human-murine somatic-cell hybrids (Nedwin et al. 1985). We have recently shown that the two genes

are very closely linked; the polyadenylation site of the TNF- β gene is separated from the transcription-initiation site of the TNF- α gene by only 1221 bp. Both have the same orientation with respect to the direction of transcription. The regions immediately flanking the two genes are extremely homologous in the human,

1			
Human	-34	MTPPERLFLPRVCGTTLHLLLGLLLVLLPGAQGLPGVGLTPSAAQTARQHPKMH LAHST	
Bovine	-33	MTPPGRLYLRLVCSTPP-LLLLGLLLALPLEAQGLRGIGLTPSAAQPAHQQLPTPFRGT	
Mouse	-33	MTLLGRLHLLRLVLTTP-VFLLGLLLALPLGAQGLSGVRF-SAARTAHPLPKHLTHGI	
Consensus		MT RL L RV T LLGLLL L AQGL G SAA A	
Human	27	LKPAHLIGDPSKQNSLLWRANTDRAFLQDGFSLSNNSLLVPTSGIYFVYSQVVFSGKAY	
Bovine	27	LKPAHLVGDPSTQDSLWRANTDRAFLRHGFSLSNNSLLVPTSGLYFVYSQVVFSGRGC	
Mouse	25	LKPAHLVGYPKQNSLLWRASTDRAFLRHGFSLSNNSLLIPTSGLYFVYSQVVFSGESC	
Consensus		LKPAHL G PS Q SL WRA TDRAFL GFSLSNNSLL PTSG YFVYSQVVFSG	
Human	87	SPKATSSPLYLAHEVQLFSSQYPFHVPLLSQKMYVPGLPQEPWLHSMYHGAFAQLTQGDQ	
Bovine	87	FPRATPTPLYLAHEVQLFSPQYPFHVPLLSAQKSVCPGPQGPWVRSVYQGAFFLLTRGDQ	
Mouse	85	SPRAIPTPIYLAHEVQLFSSQYPFHVPLLSAQKSVYPGLQGPWVRSMYQGAFFLLSKGDQ	
Consensus		P A P YLAHEVQLFS QYPFHVPLLS QK V PG Q PW S Y GA F L GDQ	
Human	147	LSTHTDGIPHLVLSPTVFFGAFAL	171
Bovine	147	LSTHTDGISHLLSPSSVFFGAFAL	171
Mouse	145	LSTHTDGISHLHSPSSVFFGAFAL	169
Consensus		LSTHTDGI HL SPS VFFGAFAL	

Figure 3. Amino acid sequences of human (Gray et al. 1984), bovine (D. Goeddel et al., unpubl.), and murine (P.W. Gray, unpubl.) TNF- β . (-34 to -1) Amino acids of the signal sequences. "Consensus" indicates the residues that are identical in all three sequences.

murine, and bovine genomes (D.V. Goeddel et al., unpubl.).

Regulation of TNF- α and TNF- β Synthesis

TNF- α and TNF- β are produced by activated macrophages (Carswell et al. 1975) and lymphocytes (Granger and Kolb 1968; Ruddle and Waksman 1968), respectively (Stone-Wolff et al. 1984). We have examined the inducibility of both TNF- α and TNF- β by mitogens on 30 different cell lines representing many cell types. As TNF- α and TNF- β behave similarly in the L-M cell cytotoxic assay, they were distinguished by neutralization of activity with the corresponding monoclonal antibodies. Northern blot analysis using TNF- α and TNF- β cDNA probes was also performed to determine which TNF was expressed.

Although normal T lymphocytes have been reported to be the cellular source of TNF- β (Ruddle et al. 1983), we have not detected any TNF activity after exposure to the mitogen PMA using two human T-cell lines (Molt-4 and Jurkat). However, all six B lymphoblastoid cell lines examined produced high levels of TNF- β . Similar results were seen by Williamson et al. (1983), but they did not distinguish TNF- α and TNF- β activities.

The majority of the human and murine macrophage cell lines tested (including HL-60, U937, PU5-1.8, RAW 264, P388D₁, J774, and WRI-7) produce TNF- α but not TNF- β when stimulated with mitogens. This result was confirmed by Northern blot hybridization with the TNF- α and TNF- β cDNA probes. Other cell types, such as normal endothelial cells, rat glial cells (C6), IL-3-dependent normal bone-marrow-derived mast cells, and a mast cell line (A1) are capable of producing TNF- α but not TNF- β . Most nonlymphoid cell lines (including HeLa, A549, T24, WI38, K562, ME-180, HT-29, MCF, SKCO-1, A-431, HT1080, 7860, NRK, Rat-1, and C127) do not produce TNFs in the presence or absence of the mitogen.

The expression of both TNF- α and TNF- β mRNAs is transient even in the presence of continuous mitogenic stimuli. TNF- α mRNA in murine macrophage PU5-1.8 cells is detectable 1 hour after induction with PMA, is highest at approximately 2–4 hours, and becomes undetectable by 12 hours. In contrast, TNF- β mRNA in the B lymphoblastoid cell line 1788 begins to increase at 4 hours, reaches its maximal level at 12–24 hours, and decreases to the basal level after 48 hours. Thus, induction of TNF- α is faster than induction of TNF- β .

The effect of physiological mediators or cytokines on the expression of TNF was also examined. Glucocorticoids, known to be immunosuppressive agents (Claman 1972; Vischer 1972), have been shown to inhibit Ia antigen expression (Wong et al. 1984) and lymphokine production (Arya et al. 1984; Culpepper and Lee 1985). We found that the glucocorticoid dexamethasone inhibited TNF- α mRNA expression in HL-60 cells (Fig. 4). Similar results were obtained by Beutler

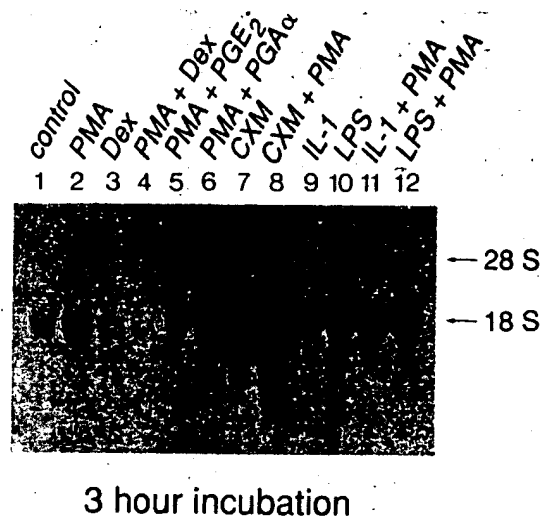


Figure 4. Regulation of TNF- α mRNA levels in HL-60 cells. HL-60 cells (5×10^6 cells/ml) were treated with PMA (50 ng/ml) in the presence or absence of dexamethasone (Dex) (5×10^{-7} M), PGE₂ (50 ng/ml), PGA α (50 ng/ml), cycloheximide (1 μ g/ml), interleukin-1 (100 U/ml), and LPS (10 μ g/ml). After 3 hr, total RNA was extracted, poly(A) RNA was prepared, and Northern hybridization was performed as described previously (Thomas 1980). Each lane contained about 1 μ g of poly(A) RNA. Hybridization was performed using a ³²P-labeled TNF- α cDNA probe.

et al. (1986) using murine macrophages. Other glucocorticoids (prenisolone, hydrocortisone, and corticosterone), but not sex steroids (testosterone, estrogen, and progesterone), inhibited the production of TNF- α in macrophage cell lines (HL-60, U937, PU5-1.8) and TNF- β in B lymphoid cells (RPMI 1788, IM9).

Prostaglandin E (PGE), which is an important modulator of inflammation and cellular immune responses (Goodwin and Webb 1980), also down-regulates the expression of TNF- α mRNA in HL-60 cells (Fig. 4), whereas prostaglandin A α or F α had no inhibitory effect. Similar inhibitory effects of PGE₁ and PGE₂ on the expression of TNF- α by other macrophage cell lines (U937, PU5-1.8, RAW 264, P388D₁, and J774) were observed. Indomethacin, a cyclooxygenase inhibitor (Sheen and Winter 1977), enhances the production of TNFs in both macrophages and B lymphoid cells. Surprisingly, PGE₁ or PGE₂ does not suppress the production of TNF- β mRNA or biological activity in two B lymphoid cell lines (RPMI 1788 and IM9). It will be of interest to examine how the differential expression of TNF- α and TNF- β is regulated by PGEs.

The protein synthesis inhibitor cycloheximide further enhances the accumulation of both TNF- α (Fig. 4) and TNF- β mRNAs induced by PMA. The production of TNFs by macrophages and lymphoid cells can also be enhanced by the lymphokine interferon- γ (IFN- γ).

Normally, LPS alone does not induce TNF- β in 1788 or IM9 cells. However, LPS in combination with PMA gave at least a tenfold greater increase in both TNF- β mRNA and biological activity than did PMA alone. Similarly, the induction of TNF- α mRNA in HL-60 cells by the combination is greater than by either in-

duction alone (Fig. 4). These results demonstrate that two different mitogens can synergistically induce the expression of both TNF- α and TNF- β genes. The regulation of the TNF genes by glucocorticoids, prostaglandins, cycloheximide, IFN- γ , and different mitogens observed in cell lines was similarly observed in normal human peripheral blood leukocytes. In addition to bacterial LPS and the mitogen PMA, both viruses and poly(I):poly(C) can induce the synthesis of TNF- α and TNF- β in TNF-producing cell lines and normal human leukocytes (Aderka et al. 1986; G. Wong and D. Goeddel, in prep.).

Antitumor Properties of TNFs

TNF is a term that was initially used to describe an activity present in the serum of bacillus Calmette-Guerin-infected endotoxin-treated mice that induced the hemorrhagic necrosis of certain transplantable tumors in inbred mice (Carswell et al. 1975). We have utilized this classic assay to compare further the ability of TNF- α and TNF- β to induce necrosis of an established Meth-A sarcoma intradermal implant in (BALB/c \times C57BL/6)F₁ mice. Intravenous injection of 1–50 μ g of either recombinant human TNF- α (r-hTNF- α) or r-hTNF- β induced significant degrees of hemorrhagic necrosis 24 hours after injection (Table 1). We have previously reported similar antitumor activity following intraperitoneal, intralesional, and intramuscular injections of TNF- α and TNF- β (Gray et al. 1984; Pennica et al. 1984).

Additional experiments were performed to characterize the in vivo antitumor activities of TNF- α against two subcutaneously implanted, chemically induced sarcomas of BALB/c origin (WEHI-164 and Meth A). Intravenous treatment with TNF- α significantly inhibited the growth of both sarcomas (Table 2). However, there were no complete remissions in the WEHI-164

group and only one of ten animals in the Meth-A group completely rejected the tumor after TNF- α treatment. The site of tumor implantation appears to be a critical factor for the demonstration of antitumor activities of TNF- α against transplantable sarcomas, since Meth A and M5076 (a spontaneously arising ovarian sarcoma of C57BL/6 origin) implanted intraperitoneally are almost completely refractory to the antitumor effects of TNF- α (M.A. Palladino, unpubl.). In contrast, treatment of the Meth-A tumor implanted intradermally results in a cure rate of almost 100%.

We have previously reported the ability of both TNF- α and TNF- β to augment superoxide radical production, antibody-dependent cellular cytotoxicity, and phagocytosis by neutrophils (Shalaby et al. 1985). The role of neutrophils in host defense responses is well established, and they have been shown to release activated oxygen intermediates (Babior 1978) and interleukin-1 (Tiku et al. 1986). Other studies have shown that neutrophils can mediate endothelial damage in vitro and that TNF- α can directly stimulate endothelial cells to produce procoagulant activity (Harlan et al. 1981; Nawroth and Stern 1986). It is therefore quite possible that the association between changes in neutrophil functions and endothelial cell homeostatic properties mediates the antitumor activities of TNF in vivo (Pennica et al. 1986). This hypothesis is supported by the fact that intraperitoneally implanted Meth A is refractory to the antitumor effects of TNF- α , whereas intradermally implanted Meth A is highly sensitive. Studies directed at addressing this hypothesis further are in progress and may give greater insight into the in vivo antitumor mechanisms of TNF.

In Vitro Growth Activities of TNFs

TNF- α and TNF- β have been shown to have comparable cytostatic and cytolytic properties in vitro (Ag-

Table 1. Necrosis of Meth-A Sarcoma after Intravenous Injection of r-hTNF- α or r-hTNF- β

Intravenous treatment	Dose (μ g)	Hemorrhagic necrosis score				Percentage of mice with >25% necrosis
		+++	++	+	–	
PBS		0	0	2	18	0
r-hTNF- α	50	15	3	1	0	95
	15	13	6	1	0	95
	5	13	5	0	1	95
	1	3	7	7	3	50
r-hTNF- β	50	14	1	4	1	75
	15	15	2	2	1	85
	5	11	5	2	2	80
	1	4	6	6	4	50

(BALB/c \times C57BL/6)F₁ female mice were injected intradermally with 5×10^5 Meth-A sarcoma cells. After 7 days (average tumor diameter 0.75 cm), TNF was injected intravenously in 0.1 ml PBS; 24 hr later, the tumors were excised, sectioned, and scored as described previously (Carswell et al. 1975; Pennica et al. 1984). +++ represents between 50% and 75% of tumor mass necrotic; ++ represents between 25% and 50% of tumor mass necrotic; + represents less than 25% of tumor mass necrotic; – represents no visible necrosis.

Table 2. Antitumor Effects of r-hTNF- α against Subcutaneously Implanted Sarcomas

Sarcoma	Intravenous treatment	Tumor size (mm) on days			<i>p</i> Value
		7	14	21	
Meth A	PBS	2.6	7.1	15.1	<0.01
	r-hTNF- α	4.3	4.3	7.6	
WEHI-164	PBS	12.4	25.2	34.9	<0.01
	r-hTNF- α	9.3	14.3	22.9	

r-hTNF- α was administered daily from day 7 to day 18 for Meth A and from day 5 to day 12 for WEHI-164 at a dose of 25 μ g/day and 15 μ g/day, respectively. There were 10 animals per group.

garwal et al. 1984, 1985b,c). In addition, IFN- γ can potentiate the antiproliferative effects of both cytokines on certain tumor cell lines (Williams and Bellanti 1983; Williamson et al. 1983; Lee et al. 1984; Stone-Wolff et al. 1984; Sugarman et al. 1985). Subsequent experiments using recombinant TNF- α and TNF- β demonstrated that they compete for the same binding sites on tumor cells, which probably accounts for their similar effects on tumor cell growth in vitro (Aggarwal et al. 1985a).

A number of studies characterizing the effects of TNF- α or TNF- β on the proliferation of various cell lines have been reported (Williamson et al. 1983; Lee et al. 1984; Kull et al. 1985; Sugarman et al. 1985; Ruggiero et al. 1986). Initial results indicated that cell lines can be subdivided into three categories on the basis of their response to TNF- α : (1) a cytostatic or cytolytic effect, (2) little or no antiproliferative effect, and (3) enhanced growth (Table 3). Furthermore, addition of exogenous growth factors can either inhibit the antiproliferative response or enhance the growth-promoting effect of TNF- α (Vilcek et al. 1986; B.J. Sugarman et al., unpubl.). More recent results have shown that tumor cells sensitive to the cytotoxic effects of TNF- α are actually growth stimulated at relatively low concentrations of TNF- α (G. Lewis et al., unpubl.).

Approximately 40% of the established tumor cell lines are sensitive to the antiproliferative effects of TNF- α (Sugarman et al. 1985). Cells such as L929, WEHI 164, and UV1591-RE are extremely susceptible to TNF- α -mediated cytotoxicity (i.e., <10 U/ml reduces cell viability by 50%), whereas a similar decrease in the viability of the most sensitive human cell lines (e.g., ME-180 and BT-20) requires a tenfold greater concentration of TNF- α . IFN- γ can enhance the cytotoxic effects of TNF- α on some TNF-sensitive cell types (e.g., ME-180 and BT-20) as well as cell lines that are insensitive to its antiproliferative effects (e.g., A549, B16F10, Saos-2, and WI38 VA13) (Sugarman et al. 1985, unpubl.). However, treatment of tumor cells with both IFN- γ and TNF- α does not always result in an enhanced cytotoxic response; no synergistic antiproliferative response is seen on T24 bladder carcinoma, Calu-3 lung carcinoma, or RPMI 7272 melanoma cell lines, which are all refractory to the cytotoxic effects of TNF- α alone (Sugarman et al. 1985; Tsujimoto et al. 1986).

The response of normal fibroblasts to treatment with

TNF- α in vitro is completely different from that of other cell lines. Their growth is stimulated in the presence of picomolar concentrations of TNF- α (Sugarman et al. 1985; Vilcek et al. 1986). An antibody that neutralizes the cytotoxic effects of TNF- α on tumor cells abrogates TNF- α -induced proliferation of normal fibroblasts (Sugarman et al. 1985). IFN- γ interferes with TNF- α -induced growth of normal fibroblasts in a dose-dependent manner (Sugarman et al. 1985; Vilcek et al. 1986). This TNF- α -induced fibroblast proliferation can also be augmented by exogenously added factors. Insulin and platelet-derived growth factor enhance the proliferation of NRK-49F fibroblasts (G. Lewis et al., unpubl.).

It is not clear how TNF- α can stimulate the growth of certain cell lines while inhibiting the growth of others. Variations in the proliferative responses induced by TNF- α are not necessarily due to differences in the number of binding sites per cell or their affinity for TNF- α (Kull et al. 1985; Sugarman et al. 1985; Tsujimoto et al. 1986). The ability of TNFs to both stimulate and interfere with cell proliferation is similar to transforming growth factor- β , a distinct growth factor/inhibitor (Tucker et al. 1984; Roberts et al. 1985), and may be characteristic of proteins involved in homeostatic regulatory mechanisms.

Antiviral Properties of TNFs

In response to viral infection in vivo, interferons (IFNs) are secreted and induce a state of viral resistance in noninfected cells (Stewart 1981). TNF- α and TNF- β also have antiviral activity on some cells and antiviral enhancing activity on most cells tested.

TNF- α and TNF- β inhibit the cytopathic effects of vesicular stomatitis virus (VSV) in murine epithelial cells (C127) and rat fibroblasts (Rat-1). In addition to inhibition of the VSV-mediated cytopathic effect, TNF- α and TNF- β also dramatically decrease the VSV yield in these cells (Fig. 5). This antiviral activity is dose-dependent and is neutralized by respective monoclonal antibodies against human TNF- α and TNF- β . The antiviral activity of TNFs is not caused by the induction of IFNs from these cells, since the antiviral activity is not abolished by polyclonal or monoclonal antibodies against IFN- α , - β , or - γ . The antiviral effects of TNF- α and TNF- β are also observed with encephalomyocar-

Table 3. Response of Various Cell Lines to TNF- α or TNF- β In Vitro

Growth Enhancement	
CCD-18Co (normal human colon)	Sugarman et al. (1985)
Detroit 551 (normal human fetal skin)	Sugarman et al. (1985)
FS-4 (normal human foreskin)	Vilcek et al. (1986)
FS-48 (normal human foreskin)	Kohase et al. (1986)
LL24 (normal human lung)	Sugarman et al. (1985)
NRK-49F (normal rat kidney)	G.D. Lewis et al. (in prep.)
Osteoclasts	Bertolini et al. (1986)
WI38 (normal human fetal lung)	Lee et al. (1984); Sugarman et al. (1985)
WI-1003 (normal human lung)	Sugarman et al. (1985)
Null response	
A549 (human lung carcinoma)	Sugarman et al. (1985)
B16 (murine melanoma)	Lee et al. (1984)
B16F10 (murine melanoma)	Sugarman et al. (1985)
Calu-3 (human lung carcinoma)	Sugarman et al. (1985)
CMT-93 (murine rectal carcinoma)	Sugarman et al. (1985)
G-361 (human melanoma)	Sugarman et al. (1985)
HeLa (human cervical carcinoma)	Sugarman et al. (1985)
HeLa D98 (human cervical carcinoma)	Ruggiero et al. (1986)
HT-29 (human colon carcinoma)	Ruggiero et al. (1986); Tsujimoto et al. (1986)
HT1080 (human fibrosarcoma)	Sugarman et al. (1985)
KB (human oral epidermoid carcinoma)	Sugarman et al. (1985)
LS174T (human colon carcinoma)	Sugarman et al. (1985)
RD (human rhabdosarcoma)	Sugarman et al. (1985)
Saos-2 (human osteogenic sarcoma)	Sugarman et al. (1985)
SK-CO-1 (human colon carcinoma)	Sugarman et al. (1985)
SK-LU-1 (human lung carcinoma)	Sugarman et al. (1985)
SK-OV-3 (human ovarian carcinoma)	Sugarman et al. (1985)
SK-UT-1 (human uterine carcinoma)	Sugarman et al. (1985)
S49 (murine lymphoma)	Sugarman et al. (1985)
T24 (human bladder carcinoma)	Sugarman et al. (1985)
WI38 VA13 (human transformed WI38)	Lee et al. (1984); Sugarman et al. (1985)
Antiproliferative response	
BT-20 (human breast carcinoma)	Sugarman et al. (1985)
BT-475 (human breast carcinoma)	Sugarman et al. (1985)
B6MS2 (murine sarcoma)	Sugarman et al. (1985)
B6MS5 (murine sarcoma)	Sugarman et al. (1985)
CMS4 (murine sarcoma)	Sugarman et al. (1985)
CMS16 (murine sarcoma)	Sugarman et al. (1985)
L929 (murine fibroblast)	Sugarman et al. (1985)
MCF7 (human breast carcinoma)	Sugarman et al. (1985)
ME-180 (human cervical carcinoma)	Sugarman et al. (1985)
Meth A (murine sarcoma)	Sugarman et al. (1985)
MMT (murine breast carcinoma)	Sugarman et al. (1985)
SAC (Moloney-transformed murine 3T3)	Sugarman et al. (1985)
SK-MEL-109 (human melanoma)	Sugarman et al. (1985)
SK-OV-4 (human ovarian carcinoma)	Sugarman et al. (1985)
UV1591-RE (murine fibrosarcoma)	Urban et al. (1986)
WEHI-164 (murine sarcoma)	Sugarman et al. (1985)
WiDr (human colon carcinoma)	Sugarman et al. (1985)

ditis (EMCV) and herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). Coincubation with the RNA synthesis inhibitor actinomycin D (1 μ g/ml) or the protein synthesis inhibitor cycloheximide (1 μ g/ml) abolishes the antiviral activity of TNFs. A 24-hour preincubation with TNF provides complete protection against VSV in C127 cells. The antiviral activity of TNF is also observed in human renal carcinoma 7860, lymphoid RPMI 8226, and murine macrophage RAW 264 cell lines. Human TNFs exhibit antiviral activity on some murine cell lines, and murine TNF- α also protects some human cells from viral infection. This suggests that the antiviral activity of TNF- α and TNF- β is not species-specific.

TNF- α or TNF- β alone protects against viral infection in some cell lines but is inactive in most cells. However, they enhance the antiviral activity of IFN- α , - β , and - γ on a variety of cell types tested. An example of their synergistic antiviral action using EMCV and the human lung carcinoma A549 cell line is shown in Figure 6. Similarly, the activity of bovine IFN- γ against VSV in bovine MDBK cells is also enhanced by human TNF- α or TNF- β .

Although IFN- γ is active in protecting most cells from VSV infection, 1 μ g/ml (10^5 units in the EMCV assay) of human IFN- γ has no detectable activity against VSV in the A549 cell line. However, IFN- γ is effective in protecting against VSV infection in the

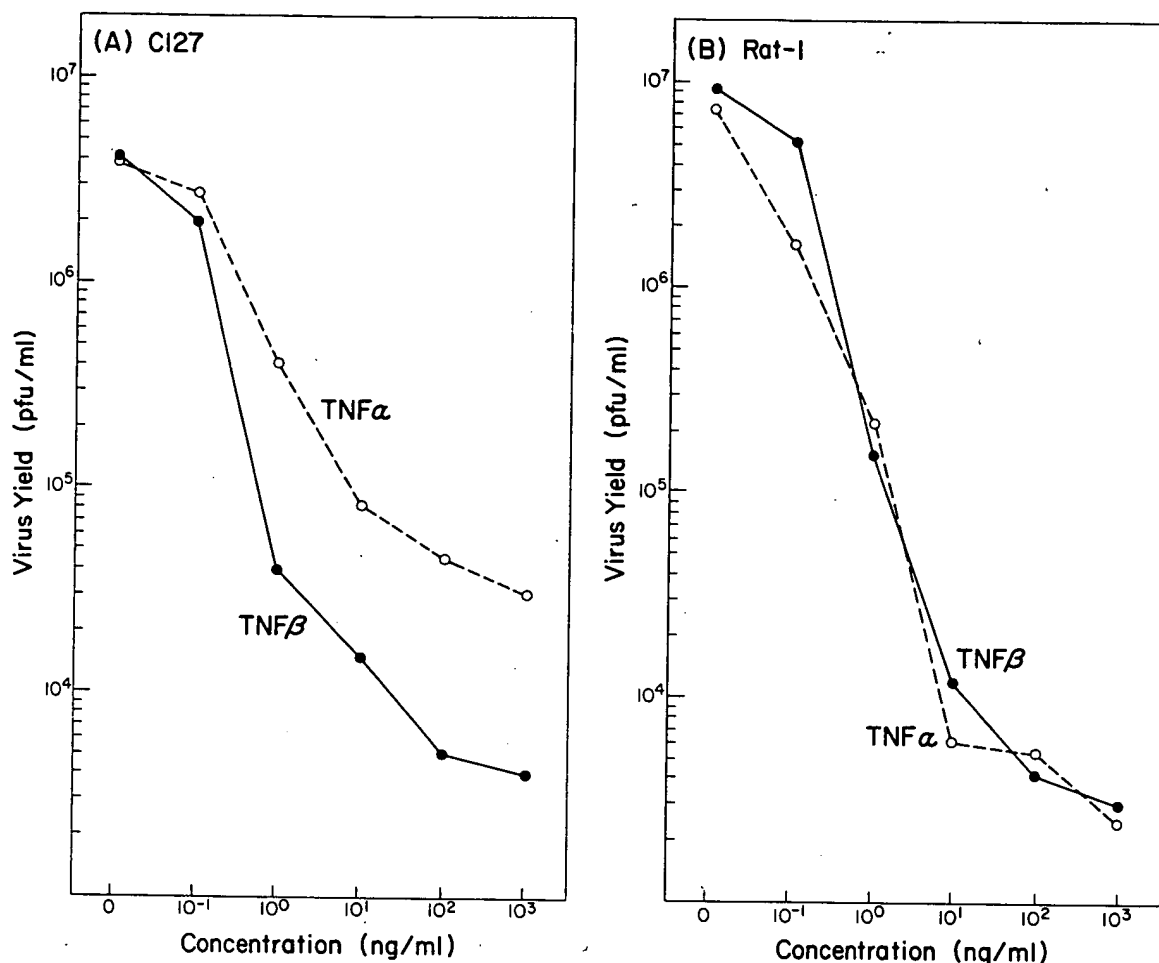


Figure 5. TNF- α and TNF- β inhibit VSV yield in murine C127 (A) and Rat-1 (B) cells. The cells were grown to confluency in 24-well plates and then treated with the indicated concentrations of TNF- α or TNF- β for 24 hr. The medium was removed before challenging with VSV at a multiplicity of infection (m.o.i.) of 10. Two hours later, the supernatants were aspirated to remove excess virus. After 24 hr, the cultures were assayed for virus yield in terms of plaque-forming units per milliliter on A549 cells (Rager-Zisman and Merigan 1973).

presence of TNF- α or TNF- β , although IFNs alone have no activity in these cells. As little as 1 ng/ml of IFN- γ and either TNF- α or TNF- β gave complete protection. Furthermore, this combination strongly inhibits the replication in A549 cells of the DNA viruses adenovirus-2, HSV-1, and HSV-2. IFN- γ alone is relatively ineffective. TNFs also enhance the antiviral activity of IFN- α and IFN- β , although to a lesser extent than IFN- γ .

TNF- α or TNF- β also enhances the antiviral activity of IFN- γ on a variety of transformed and normal cell lines. These include transformed cell lines of human (HeLa cervical carcinoma, HT1080 fibrosarcoma, 7860 renal carcinoma, T24 bladder carcinoma, HT-29 colon carcinoma, ST-486 Burkitt lymphoma, RPMI 8226 myeloma, and U87MG glioblastoma), rat (C6 glioma), and murine (C127 epithelioma and RAW 264 macrophage) origin, and three normal fibroblast cell lines (murine 3T3 and rat NRK and Rat-1). Thus, the antiviral potentiating activity of TNF- α or TNF- β is not virus-, cell-type-, or species-specific.

In addition to its preventive role against virus infection, TNF- α and TNF- β selectively kill virus-infected

cells under certain conditions. This killing is enhanced by IFN- α , - β , or - γ . Thus, one major function of TNFs may be to broaden and extend the antiviral activity of IFNs by inducing cellular resistance in uninfected cells and by selectively destroying virus-infected cells. These results have implications for the therapy of the many medically important viral diseases caused by DNA and RNA viruses.

Effect of TNFs on HLA-B7 and HLA-DR Expression

The expression of the major histocompatibility complex (MHC) is essential for the initiation and regulation of the immune response. TNFs, like IFNs, can regulate the expression of both class I (HLA-B7) and class II (HLA-DR) MHC genes. Human bladder carcinoma T24 cells express detectable levels of HLA-B7 but not HLA-DR β mRNA. Incubation with TNF- α (0.1 μ g/ml) or IFN- γ (0.01 μ g/ml) for 24 hours results in a fivefold increase of HLA-B7 but not HLA-DR mRNA. The induction is greater for the combination of IFN- γ and TNF- α than for either cytokine alone. Induction of HLA-B7 by IFN- γ is direct because an increase in

EMC/A549 Cells

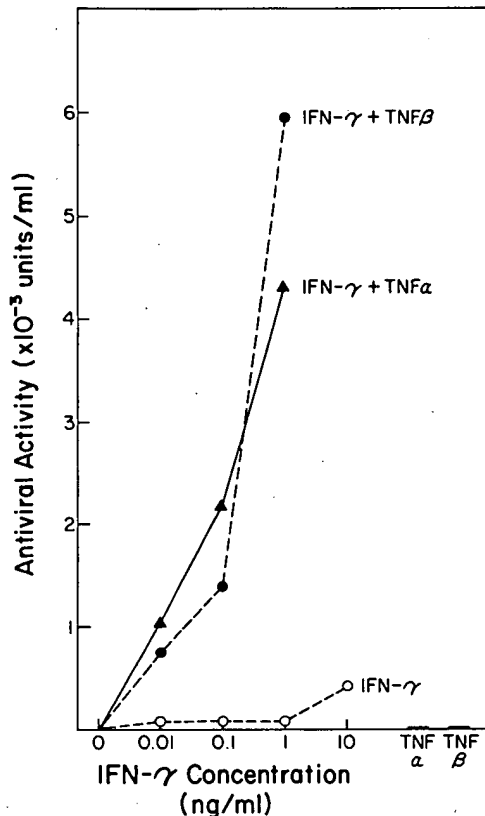


Figure 6. TNF- α and TNF- β enhance the antiviral activity of IFN- γ in A549 cells. A549 cells were grown to confluency, treated with human TNF- α (0.1 μ g/ml), TNF- β (0.1 μ g/ml), the indicated concentrations of IFN- γ , or the combinations for 24 hr before challenge with EMCV at a m.o.i. of 1. After 24 hr, the cytopathic effect was determined by staining the cells with crystal violet, and the titer was quantitatively monitored using a micro-ELISA autoreader. The assay was standardized to the International Reference Sample of the National Institutes of Health human IFN- γ (Gg 23-901-530).

HLA-B7 mRNA by IFN- γ can be detected after 4 hours of treatment in the presence of cycloheximide. In contrast, the induction of HLA-B7 by TNF- α can be blocked by cycloheximide, and the induction can only be detected after 12 hours of treatment. These results suggest that the induction of HLA-B7 mRNA by TNF- α is indirect. It is possible that TNF- α induces the synthesis of IFNs that cause the increase in HLA-B7 mRNA. However, the HLA-B7-inducing activity of TNF- α in T24 cells cannot be blocked by antibodies that neutralize IFN- α , - β , or - γ .

Although TNF- α and IFN- γ do not induce HLA-DR in most cell lines, they do induce HLA-DR β mRNA in two human monocyte-like cell lines: the promyelocytic leukemia HL-60 and the histiocytic lymphoma U937. The class-II-MHC-inducing activity of TNF- α is less potent than IFN- γ but is detectable at 24 hours. The combination of TNF- α and IFN- γ induces approximately tenfold higher levels of the HLA-DR β mRNA in these cells than TNF- α or IFN- γ alone. TNF- β also enhances the expression of both HLA-B7 and HLA-DR mRNAs in HL-60 and U937 cells.

The expression of class I and class II MHC antigens has emerged as an essential component for antigen presentation in an immune response and in the control of tumor growth in vivo (Tanaka et al. 1985). Thus, TNFs and IFN- γ may potentiate the immune response through the synergistic amplification of MHC gene expression on a variety of cell types.

Catabolic Effects of TNFs

TNF- α is thought by some to be the agent that causes cachexia or wasting in parasite-infected animals (Beutler and Cerami 1986). However, there is no direct evidence to support this hypothesis. In cultured adipocytes, TNF can inhibit the transcription of genes encoding enzymes involved in fatty acid uptake and lipid synthesis (Torti et al. 1985). This fact, coupled with the observation that the serum of wasting animals can have elevated lipid levels, has led to the TNF-cachexia theory (Beutler and Cerami 1986).

When the ability of TNF- α to inhibit lipid anabolism in adipocytes is compared with that of other cytokines, we found that this property is not unique to TNF- α but is instead a general property of many cytokines (Patton et al. 1986). In addition to TNF- α and TNF- β , IFN- α , IFN- β , IFN- γ , and interleukin-1 appear to inhibit lipid anabolism (Keay and Grossberg 1980; Beutler and Cerami 1985; Patton et al. 1986). Table 4 shows the effect of a variety of cytokines on ³H-labeled acetate uptake into lipid by 3T3 L1 fibroblasts and adipocytes. Both human TNF- α and TNF- β act across species boundaries to inhibit acetate uptake in mouse adipocytes. Murine IFN- γ exhibits 90% inhibition against mouse adipocytes, in contrast to human IFN- γ , which shows no effect. However, ³H-labeled acetate uptake is inhibited by human IFN- α (hybrid IFN- α 2/ α 1 [Bg/II]), which does exhibit cross-species antiviral activity. Uptake of ³H-labeled acetate by undifferentiated adipocytes (3T3 L1 fibroblasts) is also inhibited by the cytokines, although not as markedly (35–40%) as in the differentiated cells. Furthermore, these same cytokines inhibit lipoprotein lipase activity (the enzyme responsible for removing lipid from the serum) and stimulate the release of fatty acids into the medium (Patton et al. 1986). Thus, the net effect of cytokine action on a fat cell is catabolic; fat uptake and synthesis are inhibited and fat mobilization is stimulated (a situation that arises during starvation).

The short-term in vivo catabolic effect of cytokines is to mobilize fat into the circulation for utilization by the immune system (Beutler and Cerami 1986). If the infection becomes chronic, wasting ensues. Although exudates from endotoxin-stimulated macrophages have been reported to cause dose-dependent wasting in mice when injected over a period of days (Cerami et al. 1985), we have been unable to induce wasting in rats with repeated injections of highly purified recombinant human TNF- α . In our experiment, the initial injections cause a loss of appetite and an increase in blood lipids that lasts for about 24 hours. However,

Table 4. Effect of Cytokines on ^3H -labeled Acetate Uptake in 3T3 L1 Adipocytes and Fibroblasts

Cell type	Cytokine	pmoles acetate uptake/hr/mg protein
3T3 L1 Murine Adipocytes	Control	53.6 \pm 9.7 ^a
	r-hTNF- α	4.6 \pm 0.6 ^b
	r-hTNF- α + r-hTNF- α antibody	57.6 \pm 8.9
	r-hTNF- β	4.0 \pm 0.4 ^b
	r-mIFN- γ	5.3 \pm 0.4 ^b
	r-hIFN- γ	53.7 \pm 7.7
	r-hIFN- $\alpha 2/\alpha 1$ (Bg/II)	4.9 \pm 0.4 ^b
3T3 L1 Murine Fibroblasts	Control	8.0 \pm 0.5
	r-hTNF- α	5.2 \pm 1.0 ^c
	r-hTNF- β	4.5 \pm 0.6 ^d
	r-mIFN- γ	5.2 \pm 0.8 ^c

Cells were treated with cytokines (~ 1.5 nM) for 24 hr and then given ^3H -labeled acetate for 1 hr.

^aMean \pm S.D. ($n = 3$).

^b $p < 0.0001$ relative to control value.

^c $p < 0.005$ relative to control value.

^d $p < 0.025$ relative to control value.

appetite returned and tolerance developed by the second day (J. Patton et al., unpubl.). Comparisons between these two experiments are difficult. The fact that rapid tolerance to TNF did develop is intriguing and we are investigating this further. Another question that merits further work is whether or not the cytotoxic effect of cytokines on certain cells is simply an expression of the same catabolic effects that are seen on fat cells.

The observation that a variety of cytokines can be catabolic suggests that release of individual cytokines in the animal may be separated in location and time. Perhaps during the multiple phases of host response to infection there is a succession of cytokines appearing and disappearing in the circulation or at the site of infection. Alternatively, different infections (e.g., viral, microbial, and parasitic) or different tissues subject to the same infection may induce a different set of cytokines. Thus, if energy mobilization is to occur in many types of infection or in many sites within the body, it is critical that several cytokines with overlapping biological function share this host defense activity.

TNF Receptor Binding Studies

Both TNF- α and TNF- β interact with cells via specific cell-surface receptors (Aggarwal et al. 1985a; Hass et al. 1985; Sugarman et al. 1985). The binding is both time- and temperature-dependent, reaching a maximum within 1 hour at 37°C. The binding requires 4–6 hours to plateau at 4°C. For both cytokines, a single class of high-affinity receptors with a K_d of approximately 10^{-10} M has been identified on a variety of tumor cell lines. In a few cases (e.g., 3T3 L1 adipocytes; Patton et al. 1986), both low- and high-affinity receptors for TNF- α were observed. Most of the tumor cells examined have 1000–5000 receptors per cell.

Whether full receptor occupancy is needed for the biological response of TNF- α or TNF- β is not yet clear.

The concentration of TNF- β required for 50% killing of murine L929 cells is the same as that required to displace 50% of maximum binding, suggesting that full receptor occupancy is essential (Hass et al. 1985). However, the cytotoxic activity of TNF- α and TNF- β can be observed at severalfold lower concentrations with actinomycin-D-treated or mitomycin-C-treated cells. These metabolic inhibitors have no effect on the K_d of ligand binding, suggesting that under these conditions a very small fraction of the total receptors must be occupied for the cytotoxic response. The binding of ^{125}I -labeled TNF- α to cells can be effectively competed by unlabeled TNF- β , suggesting that both molecules are recognized by the same cell-surface receptor. This is probably a result of the structural similarity of TNF- α and TNF- β and explains their many common biological properties.

The receptor for TNF is probably a protein, since the binding of TNFs to cells can be abolished by pre-treating the cells with proteolytic enzymes. After protease removal, binding of ligand can be completely restored in 24 hours (B.B. Aggarwal and T. Eessalu, unpubl.). The binding of some peptide hormones to cell surfaces can be abolished by gangliosides (Van Heyningen 1974). However, gangliosides were found to have no effect on the binding of TNFs to the TNF receptor.

The binding of TNFs to the TNF receptor is not directly correlated with effects on cell proliferation (Sugarman et al. 1985). Cell lines on which treatment with TNF- α or TNF- β had no effect on growth (e.g., T24 bladder carcinoma) bound these ligands with the same affinity as cell lines that were highly sensitive (Fig. 7). A cell line whose growth was inhibited by TNF (ME-180 cervical carcinoma) bound to the ligand with the same affinity as cells that were growth stimulated by TNF (W138 lung fibroblasts). A similar number of receptors per cell were determined on all three cell types. The lack of correlation between binding and biological response of a given hormone has been observed previ-

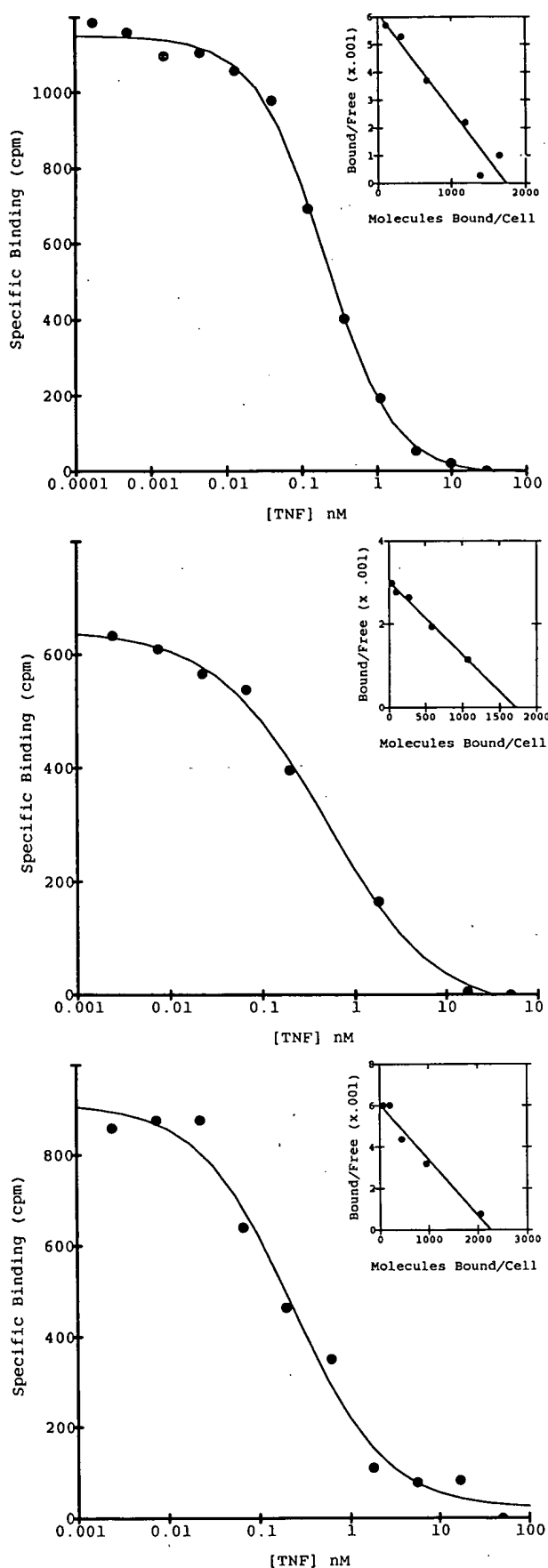


Figure 7. Competition curves of ^{125}I -labeled TNF- α with unlabeled TNF- α for binding to various cell lines. (Top) ME-180 cervical carcinoma; (middle) T24 bladder carcinoma; (bottom) WI38 lung fibroblast. (Insets) Scatchard analysis of the binding data. For experimental details, see Aggarwal et al. (1985a) and Hass et al. (1985).

ously for other proteins (Marchand-Brustel et al. 1985). This could be due to a cascade of events involved in the ultimate response of the cell to a given ligand. It is conceivable that nonresponsive cells may be defective for any one or more of these events.

We have reported that the cytotoxic activity of both TNF- α and TNF- β can be potentiated synergistically by IFN- γ (Lee et al. 1984; Sugarman et al. 1985). Although the mechanism of this synergy is not known, preexposure of cells to IFN- γ increases the total number of TNF receptors without affecting the affinity of the receptor-ligand interaction (Aggarwal et al. 1985a). A typical enhancement in TNF binding to B16 melanoma cells after exposure to IFN- γ is shown in Figure 8. Treatment of these cells for 16 hours with IFN- γ results in an approximately twofold increase in receptor number. Since this increase in TNF- α binding requires protein synthesis, it can be proposed that IFN- γ induces the de novo synthesis of TNF receptors. Whether the increase in receptor number is sufficient to explain synergistic action of TNF with IFN- γ is uncertain. Receptor induction may be just one part of the total mechanism of synergy. Since inhibitors of protein and RNA synthesis also potentiate the cytotoxic activity of TNFs, it is possible that IFN- γ also suppresses the synthesis of certain proteins that antagonize the actions of TNFs.

TNF- α and TNF- β display little species specificity. Radiolabeled human TNF- α and TNF- β bind to both human and murine cell lines, and this binding can be competed with both unlabeled murine and human TNFs. This is likely due to the close homology of human and murine TNFs (>80% amino acid sequence identity).

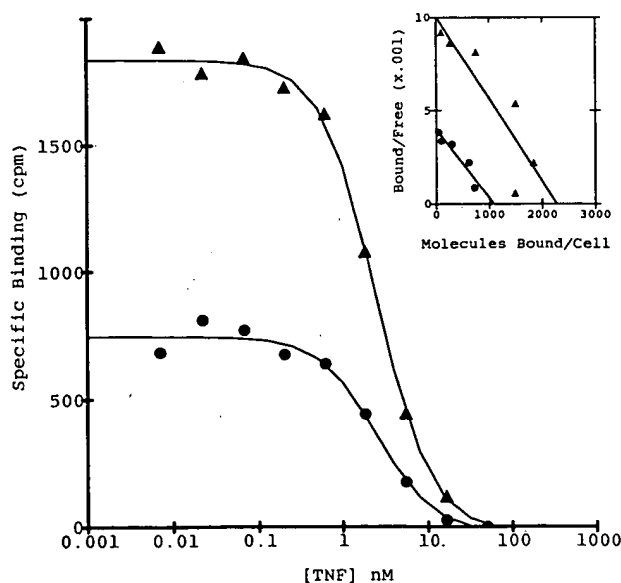


Figure 8. Induction of TNF- α receptors by IFN- γ on B16F10 melanoma cells. Competitive curves for untreated cells (●) and for cells treated overnight with IFN- γ (▲) are indicated. (Inset) Scatchard analysis of the binding data.

CONCLUSION

Our understanding of the TNF system has advanced tremendously over the past few years. The elucidation of the sequences of TNF- α and TNF- β by direct biochemical analysis and cDNA cloning has revealed that these cytokines are structurally related. Furthermore, the TNF genes are closely linked, their expression is tightly regulated, and the encoded gene products share many important biological properties. The biological activities of TNF- α and TNF- β are mediated through interaction with a common cell-surface receptor. The availability of cloned TNF genes and highly purified TNF preparations from recombinant *E. coli* should make it possible to address the many questions that remain unanswered concerning the regulation of TNF expression and the mechanisms of TNF action.

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INTERDEPENDENCE OF THE RADIOPROTECTIVE EFFECTS OF HUMAN RECOMBINANT INTERLEUKIN 1 α , TUMOR NECROSIS FACTOR α , GRANULOCYTE COLONY-STIMULATING FACTOR, AND MURINE RECOMBINANT GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR¹

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Interleukin 1 α (IL-1 α), tumor necrosis factor α (TNF α), granulocyte-colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) are molecularly distinct cytokines acting on separate receptors. The release of these cytokines can be concomitantly induced by the same signal and from the same cellular source, suggesting that they may cooperate. Administered alone, human recombinant (hr)IL-1 α and hrTNF α protect lethally irradiated mice from death, whereas murine recombinant GM-CSF and hrG-CSF do not confer similar protection. On a dose basis, IL-1 α is a more efficient radioprotector than TNF α . At optimal doses, IL-1 α is a more radioprotective cytokine than TNF α in C57BL/6 and B6D2F₁ mice and less effective than TNF α in C3H/HeN mice, suggesting that the relative effectiveness of TNF α and IL-1 α depends on the genetic makeup of the host. Administration of the two cytokines in combination results in additive radioprotection in all three strains. This suggests that the two cytokines act through different radioprotective pathways and argues against their apparent redundancy. Suboptimal, nonradioprotective doses of IL-1 α also synergize with GM-CSF or G-CSF to confer optimal radioprotection, suggesting that such an interaction may be necessary for radioprotection of hemopoietic progenitor cells.

Cytokines are hormone-like polypeptides produced by the cells of the reticuloendothelial system after inflammatory stimuli. Ample evidence exists, based on their described *in vivo* and *in vitro* activities, that these molecules serve in host defenses against harmful exogenous challenges (1, 2). Ionizing radiation, originating from natural sources (cosmic rays), represents one such environ-

mental hazard. Our previous observation that a cytokine, interleukin 1 (IL-1)² protects mice from radiation-induced death (3, 4) therefore is in accord with the concept of the role of cytokines in host defense and damage repair.

The degree of radioprotection obtained by treatment with IL-1 before irradiation resembles that previously reported for bacterial lipopolysaccharide (LPS) (5-7). Administration of LPS, however, results in induction and release of a number of cytokines, the most prominent of which are IL-1, tumor necrosis factor (TNF), and colony-stimulating factor (CSF) (8). The coordinate release of these cytokines suggests that they may act in concert. Pretreatment with human recombinant (hr) TNF α is also radioprotective, (9, 10). In contrast, we were previously unsuccessful in demonstrating radioprotection using murine recombinant (mr) granulocyte-macrophage (GM)-CSF alone (11). Furthermore, although in our hands hrIL-1 was radioprotective in five strains of mice, C57BL/6, BALB/c, DBA/1, B6D2F₁, and CDF₁, its radioprotective effect in C3H/HeN mice was minimal (12). It is possible that in the latter strain other cytokines or a combination of IL-1 with other cytokines may be more effective in radioprotection.

To evaluate the above possibilities, we have investigated the radioprotective effect of combinations of hrIL-1 α and TNF α , as well as hrIL-1 α and mr GM-CSF or hr granulocyte (G)-CSF. These studies were performed using C57BL/6 and B6D2F₁ mice, which are high responders to radioprotection with IL-1 α , and C3H/HeN mice, and low responders to IL-1-mediated radioprotection.

We now report that combinations of optimally radioprotective doses of IL-1 α and TNF α result in additive radioprotection in both high and low responder mice. Suboptimal doses of IL-1 α in combinations with nonprotective doses of GM-CSF or G-CSF result in synergistic protection from radiation-induced death.

MATERIALS AND METHODS

Mice. C57BL/6 and B6D2F₁ inbred mice were obtained from The Jackson Laboratory, Bar Harbor, ME. C3H/HeN mice were purchased from Animal Genetics and Production Branch, National Cancer Institute, Frederick, MD. The mice were housed in the Veterinary Department Facility at the Armed Forces Radiobiology Research Institute in cages with Micro-Isolation unit tops, 10 mice/cage. Fe-

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² Abbreviations used in this paper: IL-1, interleukin 1; CSF, colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; hr, human recombinant; LPS, bacterial lipopolysaccharide; mr, murine recombinant; TNF, tumor necrosis factor.

male mice 8 to 12 wk of age were used for all experiments. Standard laboratory chow and HCl-acidified water (pH 2.4) were given ad libitum. All cage-cleaning procedures and injections were carried out in a laminar flow unit.

Cytokines. The hrIL-1 α was generously provided by Immunex and Hoffmann-La Roche, Nutley, NJ. The preparations were supplied in phosphate-buffered saline, pH 7.2, and 30 mM Tris-HCl, 400 mM NaCl, pH 7.8, respectively, and used on weight basis. The hrTNF α , lot CP4026POB, specific activity 9.6×10^6 U/mg in phosphate-buffered saline, was a generous gift from Biogen Research Corp., Cambridge, MA. The mrGM-CSF was provided by Immunex as a lyophilized powder with sucrose as a stabilizing agent. The hrG-CSF was a gift from Amgen, Biochemicals, Thousand Oaks, CA. Protein-free phenol-water-extracted endotoxin derived from *Escherichia coli* K235 (LPS) was obtained from Dr. S. N. Vogel, Department of Microbiology, Uniformed Services University of the Health Sciences. All reagents were diluted to the desired concentration in pyrogen-free saline just before i.p. injection of 0.5 ml/mouse. All cytokine preparations were assayed for LPS contamination in a LAL assay and determined to contain less than 0.1 ng/inoculum.

Irradiation. Mice were placed in Plexiglas containers and were given whole body irradiation at 40 rad/min by bilaterally positioned cobalt-60 elements. Mice survival was recorded daily for 30 days.

Statistical analysis. Two survival proportions were compared using a 2×2 contingency table analysis (χ^2). A survival proportion was compared with the sum of two others by assuming that survival has an exponential distribution, i.e., $\exp(-t/\lambda)$. If two survival mechanisms act independently, their mean survival was assumed to add. The survival proportion of the combined mechanism was then compared with the predicted survival proportion of the exponential sum.

RESULTS

Comparison of the radioprotective effects of hrIL-1 α and hrTNF α . The effect of increasing doses of hrIL-1 α and hrTNF α on the survival of LD_{100/30}-irradiated C57BL/6 and LD_{95/30}-irradiated B6D2F₁ mice, both high responders to radioprotection with IL-1 α , was compared. C57BL/6 mice were protected with doses of IL-1 α ranging from 100 to 1000 ng (Fig. 1A) (doses of 50 ng did not confer significant radioprotective effect; data not shown). Doses of IL-1 α ranging from 75 to 1000 ng were similarly radioprotective for B6D2F₁ mice (Fig. 1B). Equivalent doses of TNF α had no radioprotective effect for these two strains. However, significant radioprotection in these two strains was obtained using 5- to 10- μ g doses of TNF α (Fig. 1A and B). The maximal degree of radioprotection achieved with higher doses of TNF α , however, was less than that observed in both strains with lower doses of IL-1 α ($p < 0.001$). Therefore, human TNF α is a less effective radioprotector than human IL-1 α in the above two strains.

In our previous studies, C3H/HeN mice were less responsive to the radioprotective effect of IL-1 α than C57BL/6, DBA/1 (12), as well as CDF₁ or BALB/c mice (R. Neta, unpublished observations). A comparison of the radioprotective effect of IL-1 α and TNF α in this mouse strain showed that 5.0- to 7.5- μ g doses of TNF α conferred greater protection ($p < 0.05$) than 150- to 500-ng doses of IL-1 α (Fig. 1C). Therefore, in contrast with C57BL/6 and B6D2F₁ mice, TNF α is more radioprotective than IL-1 α in C3H/HeN mice. However, TNF α is equally protective in all three strains (Fig. 1A to C). In doses of 0.2 μ g/mouse, mrTNF did not confer protection, 0.5 μ g/mouse protected 20%, 1 to 2 μ g/mouse 30%, and 5 μ g/mouse 40% of mice ($n = 10$ to 18 mice/group).

The radioprotective effects of the combinations of IL-1 α and TNF α . The divergence of the effect of TNF α and IL-1 α suggested that they may act differently. It was, therefore, of interest to determine the interactions of these two cytokines in radioprotection. The effect of com-

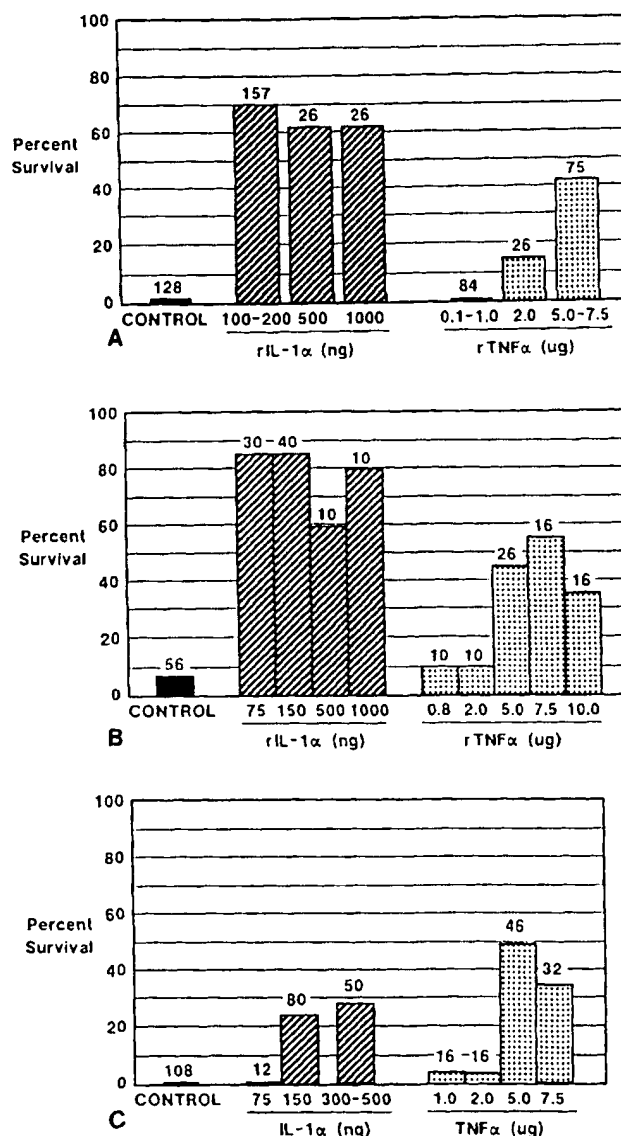


Figure 1. Protective effect of hrTNF α and hrIL-1 α in lethally irradiated mice. C57BL/6 (A), B6D2F₁ (B), or C3H/HeN (C) mice, 8 to 12 wk old, received i.p. 0.5 ml saline (control) or recombinant cytokines in doses as indicated, 20 hr before whole body irradiation. The radiation doses were 950 rad (LD_{100/30}) for C57BL/6 (A), 1050 rad (LD_{95/30}) for B6D2F₁ (B), 850 rad (LD_{100/30} for 8 to 9 wk old, and 900 rad (LD_{100/30} for 10 to 12 wk old) C3H/HeN mice (C). The numbers at the top of the bars represent the total number of mice receiving each treatment. TNF α was more radioprotective than IL-1 α in C3H/HeN mice ($p < 0.05$) (C). TNF α was less radioprotective than IL-1 α in C57BL/6 and B6D2F₁ mice ($p < 0.001$) (A, B).

binations of IL-1 α and TNF α in C57BL/6 mice was additive, as determined from the dose reductor factor (DRF) values (Fig. 2). The DRF were calculated from the ratio of LD_{50/30} of IL-1 treated to control mice. Similarly, combinations of optimal doses of the two cytokines had an additive radioprotective effect ($p < 0.01$) in lethally irradiated B6D2F₁ mice (Table I). The radioprotective effect of IL-1 α and TNF α in combination was greater in this strain than the radioprotection achieved with optimal doses of LPS ($p < 0.01$), suggesting that combinations of cytokines may be more effective radioprotectants than immunomodulatory substances that induce cytokine release.

Combinations of TNF α and IL-1 α also had additive effects in low responder C3H/HeN mice at optimal doses

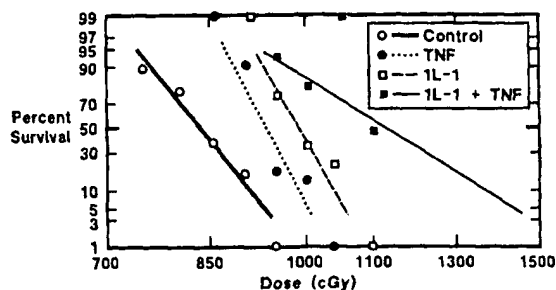


Figure 2. Radioprotective effect on hrTNF α and hrIL-1 α , by themselves and in combination, in C57BL/6 mice exposed to increasing doses of radiation. C57BL/6, 8- to 12-wk-old mice, received 5 μ g/mouse of hrTNF α , 150 ng/mouse of hrIL-1 α , alone or in combination. Each experimental point represents 12 to 70 mice. DRF were calculated from the ratio of LD_{50/30} of treated vs control mice, using probit analysis and were 1.12 (1.08, 1.16) for TNF α ; 1.19 (1.16, 1.21) for IL-1 α ; and 1.38 (1.24, 1.54) for IL-1 α + TNF α . The numbers in brackets are 95% confidence limits. Treatment with IL-1 α was significantly more radioprotective than treatment with TNF α at 950 rad ($p < 0.001$) and 1000 rad ($p < 0.05$). The effect of combined treatment with IL-1 α and TNF α was significantly greater than the sum of effects of treatment with IL-1 α or TNF α alone at radiation doses above 1000 rad ($p < 0.01$).

TABLE I

Radioprotection of B6D2F₁ mice with rIL-1 α , rTNF α , by themselves, and in combination^a

Treatment	Radiation Dose			
	1050		1150	
	Dead/Total	% Survival	Dead/Total	% Survival
IL-1 α				
100 ng	6/22	73	16/22	26
TNF α				
5 μ g	12/22	46	21/22	6
IL-1 α + TNF α				
100 ng + 5 μ g	0/22	100	4/22	82
LPS				
12 μ g	3/22	86	14/22	36
Saline	19/22	14	22/22	0

^a Mice were treated as described in Figure 1. The percentage of survival of mice given 1050 or 1150 rad after treatment with IL-1 α was greater than that after treatment with TNF α ($p < 0.05$). IL-1 α and TNF α in combination conferred significantly greater protection than the sum of radioprotection with IL-1 α and TNF α alone ($p < 0.01$) and also greater than radioprotection with optimal doses of LPS ($p < 0.01$).

TABLE II

Radioprotection of C3H/HeN mice with hrIL-1 α or hrTNF α alone and in combination^a

Treatment	Dead/Total	% Survival
Saline	175/180	2.5
IL-1 α		
100 to 200 ng	78/91	15
300 to 500 ng	48/64	25
TNF α		
1.0 to 2.0 μ g	32/34	6
5.0 to 7.5 μ g	51/88	42
IL-1 α + TNF α		
100 ng + 2.0 μ g	7/16	55
200 ng + 7.5 μ g	6/50	88

^a C3H/HeN mice were treated as described in Figure 1. TNF α in doses of 5 to 7.5 μ g/mouse protected significantly greater numbers of mice than treatment with 150 to 500 ng of IL-1 α alone ($p < 0.05$). Treatment with IL-1 α and TNF α in combination was significantly more radioprotective than the sum of the radioprotective effects of IL-1 α and TNF α administered alone ($p < 0.05$).

of cytokines ($p < 0.01$) (Table II).

The effects of combinations of IL-1 α with GM-CSF or G-CSF. The radioprotective effect of IL-1 occurs at radiation dose ranges that suppress hemopoiesis. It has been proposed, therefore, that the effect of IL-1 may be mediated by CSF. However, i.p. administration of GM-CSF 20 hr before irradiation in doses ranging from 1 to 10 μ g/mouse had no significant protective effect against lethal

doses of radiation (11). To examine further whether GM-CSF contributes to radioprotection, suboptimal doses of IL-1 α were administered in combination with GM-CSF or G-CSF. Combinations of these cytokines greatly enhanced the survival of mice in comparison to the effect of each cytokine alone ($p < 0.01$) (Table III). The effect of treatment with combinations of suboptimal doses of IL-1 α and GM-CSF or G-CSF equaled that achieved with optimal doses of IL-1 α . This effect, however, did not extend to supralethal doses of radiation (Table III).

DISCUSSION

Inflammatory signals induce the release of various cytokines with distinct, as well as overlapping, biologic activities. IL-1 α and TNF α represent two such cytokines, which are induced and released by macrophages after the same inflammatory stimulus (LPS as an example) and which also share a number of similar biologic properties, such as induction of fever (13, 14), acute phase proteins (15, 16), or CSF (17-19). Therefore, despite their differing molecular structure and their action on separate receptors, they exhibit apparent redundancy. Our results showing that the two cytokines differ in the extent of their radioprotective effect, that their relative effectiveness may vary depending on the genetic makeup of the host, and that their combined activity is additive independent of the genetic makeup argue against the apparent redundancy of these agents in this context. Higher quantities of TNF α than of IL-1 α were required in all strains of mice to confer optimal radioprotection. Although most of these studies utilized hrTNF α , murine TNF α in C3H/HeN mice was also required in doses higher than IL-1 to achieve significant radioprotection.

The radioprotection achieved with optimal doses of IL-1 α was greater than that with optimal doses of TNF α in C57BL/6 and B6D2F₁ mice, but this situation was reversed in C3H/HeN mice. TNF α , however, was equally protective in all three strains. Although we do not know the basis for the differences in protection of these inbred strains of mice, this observation suggests that different cytokines may achieve similar effects in genetically disparate individuals.

The additive effect of IL-1 α and TNF α in radioprotection, independent of genetic makeup or of the dose, sug-

TABLE III
Radioprotection of B6D2F₁ mice with combinations of rCSF and rIL-1 α ^a

Treatment	Radiation Dose			
	1050		1150	
	Dead/Total	% Survival	Dead/Total	% Survival
IL-1 α				
100 ng	9/32	69	16/22	26
33 ng	34/42	19	20/22	9
GM-CSF				
1 μ g	35/42	17	22/22	0
G-CSF				
1 μ g	8/10	20	ND	
GM-CSF				
1 μ g + IL-1 α 33 ng	14/42	67	20/22	9
G-CSF				
1 μ g + IL-1 α 33 ng	3/10	70	ND	
Saline	35/42	17	22/22	0

^a Mice were treated as described in Figure 1. The radioprotective effect of 33 ng IL-1 α or 1 μ g of GM-CSF or G-CSF did not differ significantly from treatment with saline. The effects of IL-1 α and GM-CSF or IL-1 α and G-CSF in combination in mice treated with 1050 rad differed significantly from controls ($p < 0.01$). ND, not determined.

gests that the two cytokines employ different radioprotective pathways. Although the mechanism of action to achieve radioprotection remains unknown, a number of the activities of IL-1 α and TNF α may be related to the radioprotective effect. For example, induction of acute phase proteins, some of which (metallothionein and ceruloplasmin) have the capacity to scavenge free radicals (20–23) and other acute phase proteins, may contribute to radioprotection. Although IL-1 α induction of bone marrow cell cycling (24) may present yet another critical event in radioprotection, TNF α is not known to have this capability. In fact, TNF α has been reported to be inhibitory to hemopoiesis (25, 26). Several reports exist, however, showing its role in hemopoietic differentiation (27–29). This differentiating effect is most pronounced in synergy with other cytokines. Whether this effect of TNF α on hemopoietic cells contributes to its radioprotective effect remains to be established.

The finding that treatment with TNF α and IL-1 α in combination is more effective than treatment with optimal radioprotective doses of LPS (an inducer of the two cytokines) may be explained in two ways. Either the two cytokines are presented in more optimal doses than can be induced with LPS or toxic effect of the LPS molecule itself is circumvented by using the cytokines.

The lack of radioprotective effects of GM-CSF or G-CSF administered alone, and its synergistic effect when combined with suboptimal doses of IL-1 α , indicate that these hemopoietic growth factors may be effective only when combined with IL-1 α . Possibly, this synergy relates to the recently described hemopoietin-1 (HP-1) activity of IL-1 α , because hemopoietin-1/IL-1 has been reported to synergize with GM-CSF in promoting growth of early hemopoietic progenitor cells (30). Furthermore, IL-1 α has been shown to induce CSF in vitro as well as in vivo (17, 19). Thus administration of IL-1 α generates cytokines with which IL-1 α can interact to yield more pronounced biologic effects. Additional possibilities that need to be examined may involve induction by IL-1 α of increased expression of CSF receptors.

In all, our observation that combinations of cytokines may be more effective than the administration of each cytokine alone serves as additional evidence that these agents act in concert and despite their apparent redundancy must all be required for normal host defenses.

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